CHAPTER IV

RESULTS AND DISCUSSION

1. Molecular weight of pectin powder

The molecular weight of three types of pectin were investigated from gel permeation chromatography (GPC) as presented in Table 2. GPC is one of the most widely used techniques to determine the molecular weight and molecular weight distribution of polymeric materials. Dynamic light scattering and intrinsic viscosity are also used to determine polymer molecular weights, but GPC is the only one that separates the polymers molecules by their size. Using the GPC method, the molecules are separated by introducing a dilute solution of polymer to a column packed with little tiny bead (a polystyrene gel crosslinked with divinylbenzene). The bead contains pores of different sizes (i.e. $10^3 - 10^7$ Å diameter). Larger particles pass through the columns first, while the smaller molecules become hung-up in the pores of the column resulting in longer retention times because smaller molecules can fit into more pores than larger molecules. The retention volume, which is used to characterize the polymer, is calculated from the flow rate, f, (constant experimental parameter) and the retention time, τ. The retention volume is the volume of the polymer solution that passed through the column to bring the molecule of one particular size to the detector (Sandler et al.,1998; DeRosa,2008). The retention volume(V_r) can be calculated using equation 7.

$$Vr = f * \tau \qquad (7)$$

The molecular weight of pectins were affected by the purification method. It was reported by Yapo that pectin isolated by metal ion-precipitation procedure had lower molecular weight than alcohol - precipitaion procedure and dialysis procedure. (Yapo,2009) and related to viscosity (Choukchou-Braham et al.,2003).

Three types of pectin had different molecular weight as shown in Table 2 may be due to pectins are an extremely complex and structurally diverse group of polymers. The fine structures of pectins can be extremely heterogenus between plants, between tissues and even within a single cell wall. However, all pectins are rich in galacturonic acid as 'backbone' and have neutral sugar as 'side chain' (Willats et al.,2006). The molecular weight distribution of four commercial pectins analyzed by gel permeation chromatography in-line with multi-angle light scattering and refractive index detectors ranged from $8x10^3$ to 10^6(Corredig et al.,2001). The molecular weight of pectic polysaccharide was comparable with previously reported values for pectin extracted from durian rinds which was ranged from 21,000 to 1,260,000 Da (Pongsamart et al.,2001). The intrinsic viscosity [η] is influenced by the so-called hydrodynamic volume of a macromolecule in solution. It reflects of size or physic-chemical properties of the molecule that depends primarily on the molecular weight, chain rigidity and solvent quality due to the Mark-Houwink-Kuhn-Sakurada equation (Equation 8) (Yapo,2009).

$$[\eta] = KM_v^a \quad (8)$$

Where [η] and M_v are the intrinsic viscosity and viscosity-average-molecular weight of the polymer, respectively and K and a, the Mark-Houwink constants for a given polymeric solute-solvent system.

It has been reported that the molecular weight of polysaccharides strongly affected their functionality such as gelling properties. In addition, intrinsic factors (degree of esterification, presence of neutral sugar and average molecular weight) and extrinsic factors (pH, salt concentration and pectin concentration) influence pectin functional properties (Corredig et al.,2001; Yapo,2009). Moreover, the molecular mass of polymer influenced its bioadhesion characteristics which was a necessary property for nasal delivery (Ugwoke et al.,2001).

Table 2 The molecular weight of three types of pectin using GPC

Type of pectin	Pectic polysaccharide	HM-Pectin	LM-Pectin
Weight average molecular weight, M_w (Da)	237,595	246,198	309,170
Polydispersity	2.82	2.72	2.76

2. Preparation of spray dried pectic polysaccharide microparticles

The spray drying is an one step process and it was not difficult to carefully disperse pectin powder into water and stir overnight to be completely dissolved solution. The outlet temperature was affected by inlet temperature, feed rate and aspirator rate. It increased as feed rate decreased and decreased as aspirator rate decreased as shown in Table 3. The outlet temperature was not a stable value but it could be more or less about 1-2°C. The obtained spray dried product was fine powder and had off white color.

3. Physical properties of spray dried pectic polysaccharide microparticles

3.1. Selection of optimum concentration

From data, the pectic polysaccharide concentration in feed solution affected the particle size of obtained powder. It was found that particle size (volume median diameter ranged from 6.08 to 13.54 μm) of the spray dried pectic polysaccharide particles increased as pectic polysaccharide concentration concentration was increased and leveled off at concentrations higher than 2% w/v (Figure11). The results corresponded to the finding of Elversson on spray dried lactose that the relationship between solids content and particle size of spray dried lactose which was positive but leveled off at the concentration higher than 5% w/w (Elversson et al.,2003; Elversson et al.,2005).

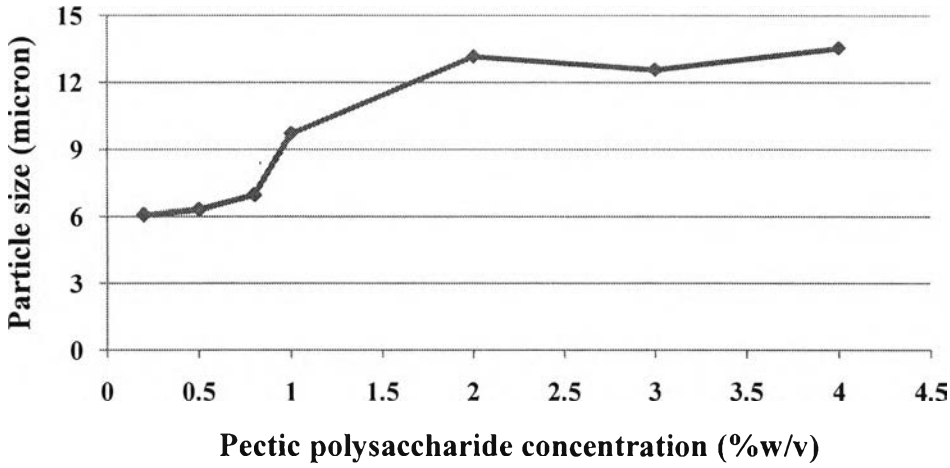


Figure 11 The relationship between pectic polysaccharide concentration and particle size of spray dried pectic polysaccharide powder without any additives at the processing condition including inlet temperature of 120°C, 80% aspirator, feed rate 3 ml/min (n=1)

The results may be explained that the solution was pumped through an atomizer a plume of liquid droplets containing molecules of solid components was created and subsequently exposed to a suitable gas stream to promote rapid evaporative mass transfer of the liquid carrier into the gas. When sufficient liquid mass had been transformed to vapor, the remaining substance in the droplet formed an individual dried particle which was then separated from the gas stream (Snyder,2008). At low feed concentration, low amount of substance was in each droplet, thus causing the droplet to dry to a small particle (Mosén et al.,2004). Therefore the pectic polysaccharide concentration of 0.2% was selected to use in the next experiments due to the obtained smallest particle size of 6 μm which is suitable for nasal application.

3.2 Selection of optimal preparing conditions

The average particle size of all obtained spray dried powders varied from 5.44 – 8.32 μm (Table 3) and the size distribution was considered to be narrow (span value < 2). In addition, decreasing the inlet temperature seemed to slightly decrease the particle size

for both feed rates of 3 and 5 ml/min (Figure 12-13) except that from the formulation of PP+MT+A+PG the condition of 90°C, aspirator rate of 80% and feed rate of 3 ml/min it was reported that the particle size of spray dried powder was highly dependent on the material being dried (Broadhead et al.,1992), solid content and feed rate.

Table 3 Effects of spray drying parameters on physical properties of pectic polysaccharide microparticles in the formulation of PP+MT+A+PG by using 0.2% PP

Spray drying condition			T_{outlet} (°C)	Particle size (span)**	Yield (%)
T_{inlet}(°C)	Feed rate (ml/min)	Aspirator rate (%)			
120	5	90	63	6.67 (1.40)	50.00
		80	63	7.33 (1.54)	28.78
	3	90	69	7.69 (1.23)	52.87
		80	69	8.31 (1.31)	51.01
120 *	3	90	69	5.44 (1.93)	48.12
110	5	90	60	6.94 (1.50)	46.62
		80	60	6.44 (1.66)	31.08
	3	90	65	7.88 (1.25)	52.12
		80	62	6.69 (1.78)	41.22
100	3	90	60	6.58 (1.69)	53.72
		80	57	6.19 (1.99)	46.75
90	5	90	45	5.56 (1.47)	33.02
	3	90	54	5.79 (1.73)	47.17
		80	53	7.19 (1.61)	39.53

* The components of this formulation did not include propylene glycol, **n=3

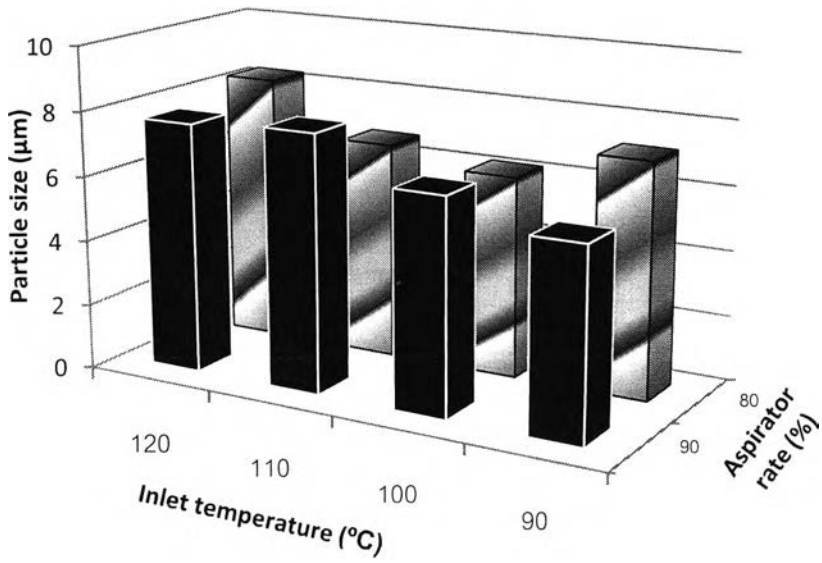


Figure 12. The effects of inlet temperature , and aspirator rate on particle size of microparticles in the formulation of PP+MT+A+PG by using 0.2% PP at feed rate of 3 ml/min.

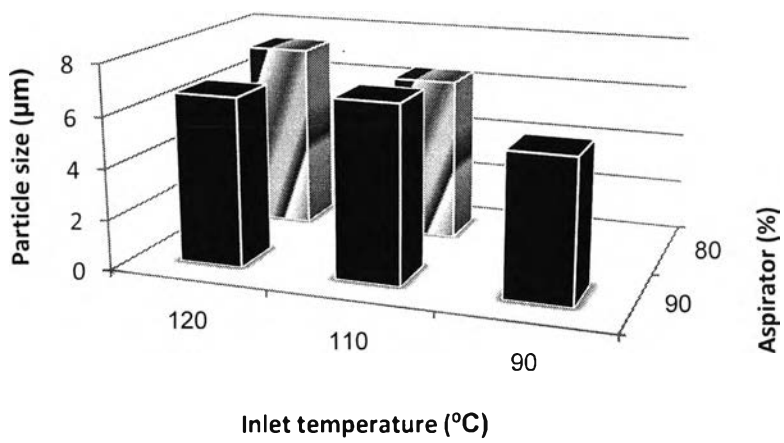


Figure 13. The effects of inlet temperature , and aspirator rate on particle size microparticles in the formulation of PP+MT+A+PG by using 0.2% PP at feed rate of 5 ml/min.

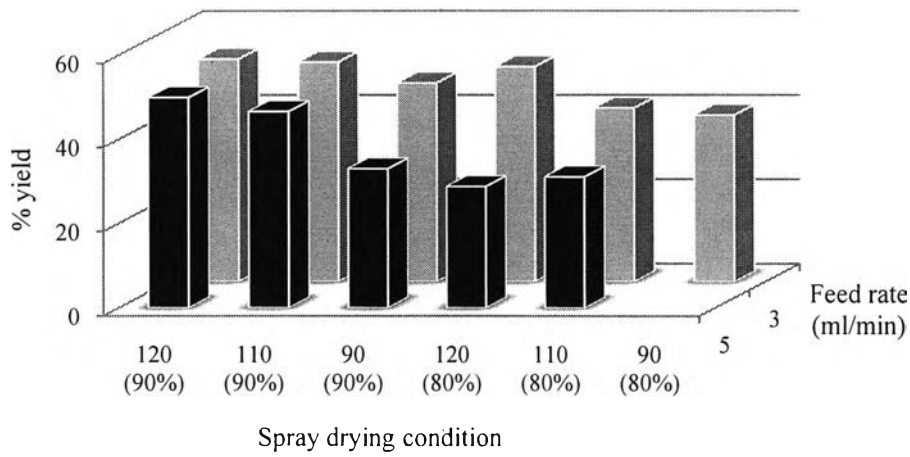


Figure 14. The effects of inlet temperature , aspirator rate and feed rate on production yields (n=1) of spray dried microparticles in the formulation of PP+MT+A+PG by using 0.2% PP

The production yield of each spray drying condition was ranged between 28–53% (Table3). In this study, the yields of spray drying process were relatively low. This might be related to the difficulty encountered in the recovery of the final product in the spray drying process and more particularly with lab scale spray dryers (Benchabane et al.,2007). There are two main reasons for a low powder yield obtained on the Büchi. Firstly, the design of the cyclone separator cannot trap particles of diameter < 2μm, but lets them pass through into the outlet air (Maa et al.,1998). Secondly, inadequate process conditions cause particles to adhere to the inside wall of the spray dryer (Maury et al.,2005).

Pectic polysaccharide powder yields were under the influence of the simple effect of the aspirator rate, inlet temperature and feed rate. The production yields were higher with aspirator rate (drying air flow rate) of 90% than 80% (Figure 14) because higher drying air flow rate resulted in increasing rate of separating powder from air in the

cyclone. The drying of a wet droplet proceeds in two phase; (i) a period where the drying rate is constant, followed by (ii) a region of steadily decreasing drying rate. During the period of constant drying rate, the temperature at the droplet surface is maintained at the wet bulb temperature (T_{wb}), corresponding to 100% relative humidity.

The drying rate of a droplet in the drying chamber can be quantified based on the temperature difference (ΔT) between the air temperature (T_{air}) and the droplet surface temperature (T_s)

$$\Delta T = T_{air} - T_s \tag{8}$$

In principal, T_s is the wet bulb temperature (T_{wb}). Since T_{air} decreases along the drying chamber due to continuous evaporation and heat loss to the surroundings, it is reasonable to use arithmetic mean (AMTD) of ΔT's at the inlet and the outlet for calculation.

$$AMTD = (\Delta T_0 - \Delta T_1)/2 \tag{9}$$

$$\Delta T_0 = T_{inlet} - T_{wb} \tag{10}$$

$$\Delta T_1 = T_{outlet} - T_{wb} \tag{11}$$

Thus, a decrease in both T_{inlet} and T_{outlet} results in a lower ΔT and drying rate. Inlet air temperature, T_{inlet}, is the one of the four independent operating variables in the spray-drying process whereas outlet air temperature, T_{outlet}, is affected by four variables : T_{inlet} , the liquid feed rate, the drying air flow rate and the atomizing air flow rate. (Maa et al.,1998)

A higher inlet temperature increased the outlet temperature proportionally and offered a dryer product, which was less sticky if inlet temperature was not more than T_g of substances and increased the yield. Also, lower feed rate offered a dryer product,

which was less sticky as shown in Table 3 and Figure 14. Therefore higher inlet temperature and/or lower feed rate affected higher production yield. This result was comparable to the research of the production of low molecular weight heparin- loaded polymeric microsphere by spray drying which had highest yields (56%) with highest inlet temperature (100°C) (Motlekar et al.,2008). Moreover, Broadhead et al. reported that during spray drying of β-galactosidase the yield was increased with increased inlet air temperature and decreased feed rate (Broadhead et al.,1994). However, the selected condition was inlet temperature of 120 °C, 90% aspirator and feed rate of 5 ml/min because of high percentage production yield of 50.00%, suitable outlet temperature for stability of protein [midpoint denaturation temperature of BSA~65°C (Ortbauer et al.,2008)] [denature temperature of insulin ~65°C (Ståhl et al.,2002)] and suitable particle size (5 -7 μm) (Arora et al.,2002) and narrow size distribution appropriate for nasal delivery. Although the preparing condition including inlet temperature of 120°C, 80% aspirator and feed rate of 3 ml/min resulted higher production yield, this condition made the process more time consuming due to lower feed rate.

3.3 Selection of optimal formulation

After selection of spray drying condition, various formulations were prepared. The results in Table 4 showed that the production yield of PM (24%) less than PL (45.50%) thus PL was selected to use in the next experiment. This result probably due to the lower T_g of mannitol [13°C;(YU et al.,1998)] affected on mannitol to be rubber y state at drying temperature and sticky to cyclone wall.

From these formulations, the particle size was 5.23 – 17.40 μm, confirming that the powders obtained were microparticles. And the largest volume weight mean diameter (particle size) and widest size distribution was obtained from the formulation of PM as a result of the formation of bridge between the particle at temperature higher than T_g.

Table 4 Effects of formulation on physical properties of spray dried pectic polysaccharide microparticles at the preparing condition including inlet temperature of 120 °C, 90% aspirator and feed rate of 5 ml/min

Formulation	Yield (%)	Size (μm)	Span	Compressibility* (Carr's Index)	Bulk density* (g/ml)	Moisture content* (%)	Moisture adsorption *,** (%)	Swelling index*	Mucoadhesive force *(N)
PM	24.00	17.40	4.22	33.14 ± 3.59	0.160 ± 0.002	3.43 ± 0.08	9.80 ± 0.07	22.41±2.51	5.52±1.11
PL	45.50	5.23	1.75	34.08 ± 4.05	0.195 ± 0.022	5.48 ± 0.18	11.34 ± 0.34	21.00±4.72	5.65±0.47
PLA0.1	51.50	5.42	3.47	45.28 ± 2.63	0.164 ± 0.004	4.88 ± 0.23	10.66 ± 0.26	25.00±5.00	5.99±0.51
PLA0.12	51.28	8.95	1.15	43.59 ± 1.09	0.207 ± 0.02	6.74 ± 0.18	14.19 ± 0.27	45.92±7.70	8.11±0.75
PLA0.15	55.60	9.83	1.99	38.74 ± 2.41	0.201 ± 0.004	4.63 ± 0.15	10.53 ± 0.45	27.78±7.86	5.34±0.71
PLA0.2	37.70	10.53	2.47	31.59 ± 5.35	0.288 ± 0.033	4.87 ± 0.16	14.80 ± 0.07	31.94±1.20	6.62±1.27
LM-PLA0.12	38.50	13.98	1.85	34.10 ± 1.76	0.196 ± 0.003	5.55 ± 1.28	13.13 ± 0.04	54.76 ± 4.12	14.45±1.12
HM-PLA0.12	47.36	10.95	1.36	37.42 ± 1.90	0.232 ± 0.016	4.97 ± 0.33	10.13 ± 0.15	24.52±4.31	10.44±1.45
P+MT+A	48.10	5.43	1.93	27.05 ± 2.13	0.197 ± 0.009	6.33 ± 1.21	11.96 ± 0.20	25.54±3.48	6.27±0.57
P+MT+A+PG	48.12	6.67	1.40	26.99 ± 1.50	0.200 ± 0.002	5.95 ± 0.23	12.96 ± 0.30	13.90±5.08	5.18±0.08

P : pectic polysaccharide; *n=3 ; **: moisture adsorption at time of 48 hrs.

Thus, lactose was selected to further add Aerosil® in the formulation. The larger particle size was obtained with increasing the quantity of colloidal silicon dioxide (Table 4 and Figure 15). The production yield increased with increasing the amount of colloidal silicon dioxide since producing powders without strong adhesion on the walls seems to be related to microparticles cover by the silica particle (Tewa-Tagne et al.,2006). However, increasing the colloidal silicon dioxide concentration to 0.2%, the production yield decreased to lower than the formulation without colloidal silicon dioxide because of the viscosity increase and as result a poor atomization aptitude causing the yield reduction. The production yield and particle size of the formulation of P+MT+A with/without Aerosil® were not quite different from the formulation of PL this may be due to high T_g of maltodextrin leading to high T_g of compounds. The production yield of all obtained spray dried powder from both commercial LM and HM pectin were lower than pectic polysaccharide and particle size of all obtained spray dried powder from both commercial LM and HM pectin were larger than pectic polysaccharide.

A great volume reduction under tapping is correlated with poor flow properties powders. It has been experimentally suggested that a material with a Carr's index above 25% has as a poor flow properties (Gabaude et al.,2001). The flowability of spray dried powder in the formulation of P+MT+A with/without PG were better than the other formulations in this research. The better flow property of spray dried powder was observed at formulations with increasing in the colloidal silicon dioxide concentration.

From all obtained spray dried microparticles, it was found that the bulk density was not quite different at various formulations but the bulk density of the formulation containing the highest amount of colloidal silica (Aerosil®) was highest due to that colloidal silica is well known as spray-drying adjuvant mainly for increasing particles densities (Palmeri et al.,1994) This result was consistent with the previous report of Tewa_tagne et al. (Tewa-Tagne et al.,2006)

Base on the results in Table 4 and Figure 15, the spray dried powder produced by the mini spray dryer by using a T_{outlet} not more than 65 °C had the residual moisture content ranged from 3.4 to 6.7% and the driest powder was obtained from the formulation of PM. The final moisture level of the solid is determined by two factors, the nature of the material and the humidity of the drying condition (Maa et al.,1998). It has been reported that the driving force for spray drying is the difference in vapor pressure between the drying air and the droplet surface. The drying air was determined by the absolute humidity of the air before entering the chamber and by the inlet air temperature (Maa et al.,1998).

The swelling property related to moisture adsorption which is a mechanism of swelling of polymer by adsorption of water before (Figure16 and Table4). The formulation containing LM pectin had the highest swelling index due to low degree of esterification. This result probably due to that when the hydrophilic group (-COOH) was replaced by the hydrophobic group ($-OCH_3$), the extent of intermolecular hydrogen bonding decreases. The swelling index and moisture adsorption of spray dried microparticles were increased as the amount of Aerosil® was increased until to the Aerosil® amount of 0.12%. These results may be due to the thickening agent of Aerosil®.

Pectin has many hydroxyl (-OH) groups and carboxyl groups (-COOH) to form hydrogen bond with functional group in mucus layer. From data, the mucoadhesive force of all obtained microparticles was ranged from 5 – 15 N which higher than the previous study of Thirawong et al. on the mucoadhesive properties of various pectins on gastrointestinal mucosa (Thirawong et al.,2007). The mucoadhesive force of increasing amount of colloidal silicon dioxide were not significantly different ($p<0.05$, ANOVA) because silicon dioxide did not have mucoadhesive property as shown in Figure 16. In this study, the higher mucoadhesive force of low methoxylated pectin (LM pectin) as shown in Table 4 was possibly due to its higher net negative charges and higher molecular weight of LM pectin than those of HM pectin and PP (Liu et al.,2005; Thirawong et al.,2007).

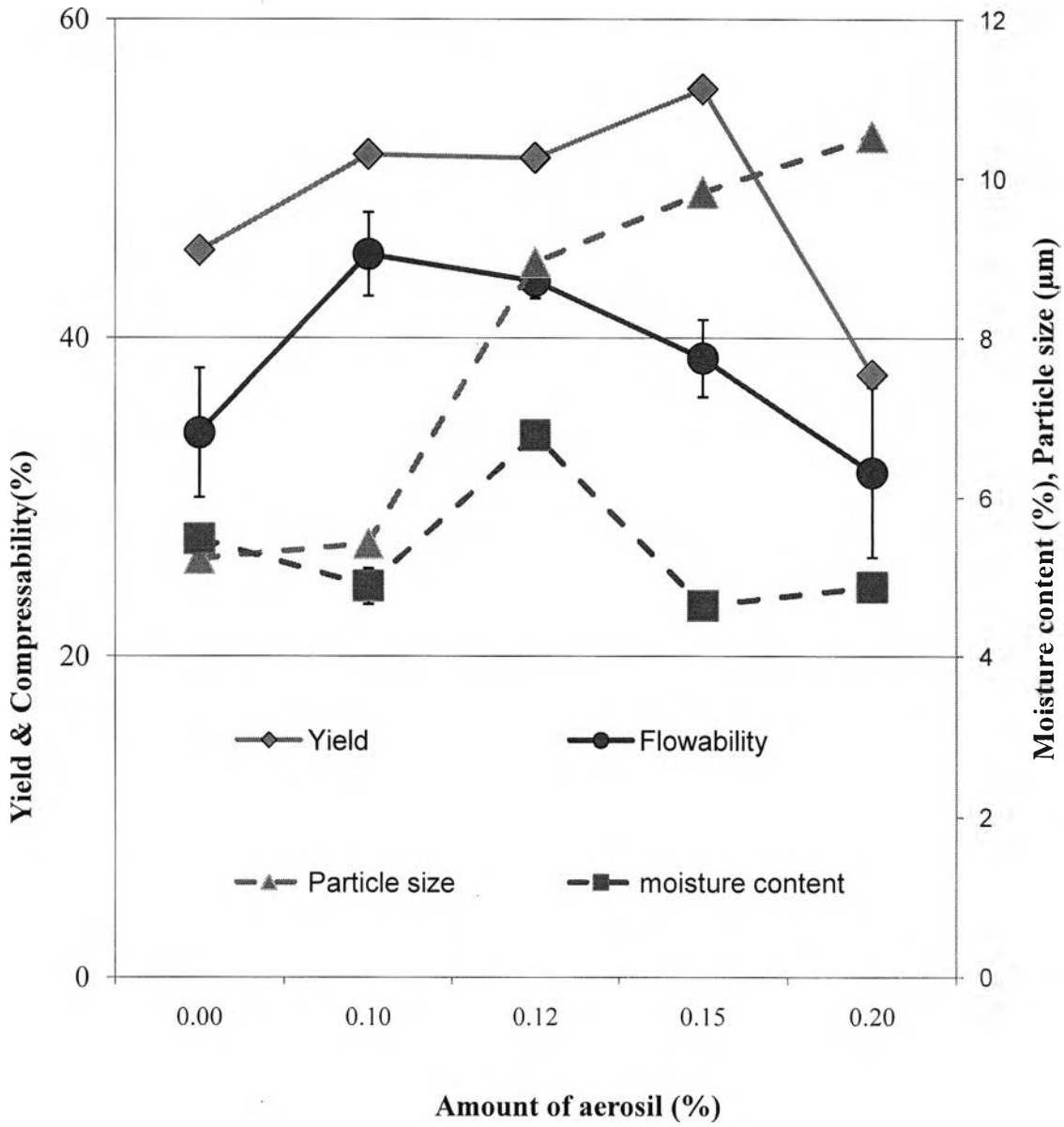


Figure 15. The relation between amount of Aerosil® flowability, moisture content and yield of pectic polysaccharide microparticles in the formulation of PL and various amount of A (n=3) Solid line was %yield and %compressability (Carr's index) and swelling index. Dashed line was moisture content and particle size.

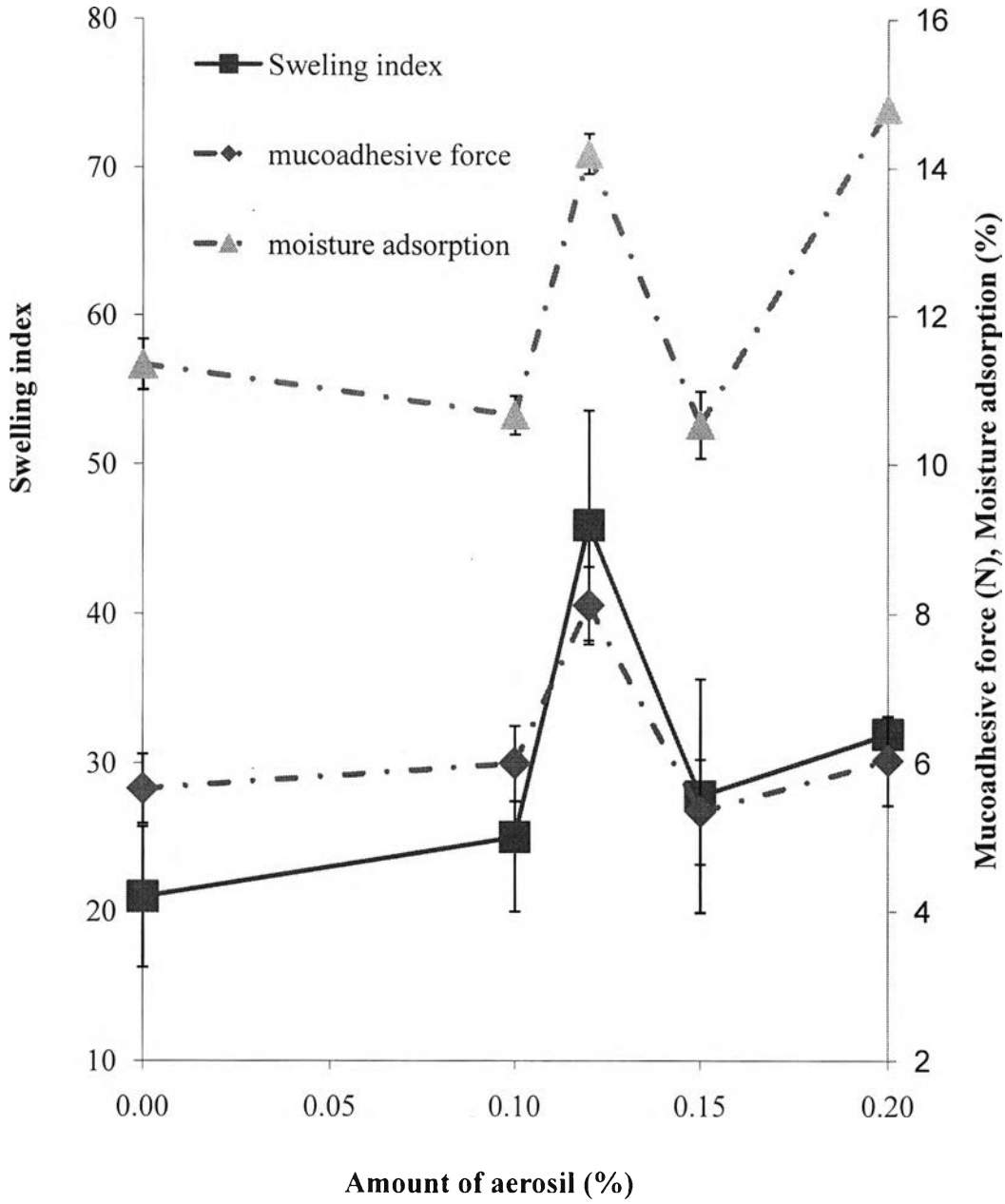


Figure 16. The relation between amount of Aerosil® and mucoadhesive force, swelling index , moisture adsorption of pectic polysaccharide microparticles in the formulation of PL and various amount of Aerosil® (n=3) Solid line was swelling index. Dashed line were mucoadhesive force and moisture adsorption

From the Table 4 and Figure 18, data of moisture adsorption calculated from percentage weight gain after keeping in the 75 % relative humidity desiccator for 48 hours showed that the formulation of PM had the lowest value. These results may be due to that mannitol has the property to resist moisture sorption, even at high relative humidity (Rowe et al.,2003). For the formulations including pectic polysaccharide and lactose, the data from Figure 17 showed the immediate uptake of moisture by lactose followed by weight decrease as reported by Shawqi Barham. These results may be due to crystallization of lactose. As crystallization proceeds there is a tendency to expel excess moisture. (Shawqi Barham et al.,2006). Whereas the formulation of PP and lactose with increasing amount of Aerosil® tended to lower the weight decrease until stable at the amount of Aerosil® 0.2% . These results could explain that colloidal silicas have a large surface area and highly polar silanol surface, which imparts a high moisture adsorption capacity. Water adsorbs onto the surface of colloidal silicas by forming hydrogen bonds with silanol (Wen,2007). In addition, the formulation of both commercial LM and HM pectin had similar trend of the moisture adsorption at various times to pectic polysaccharide as shown in Figure 18.

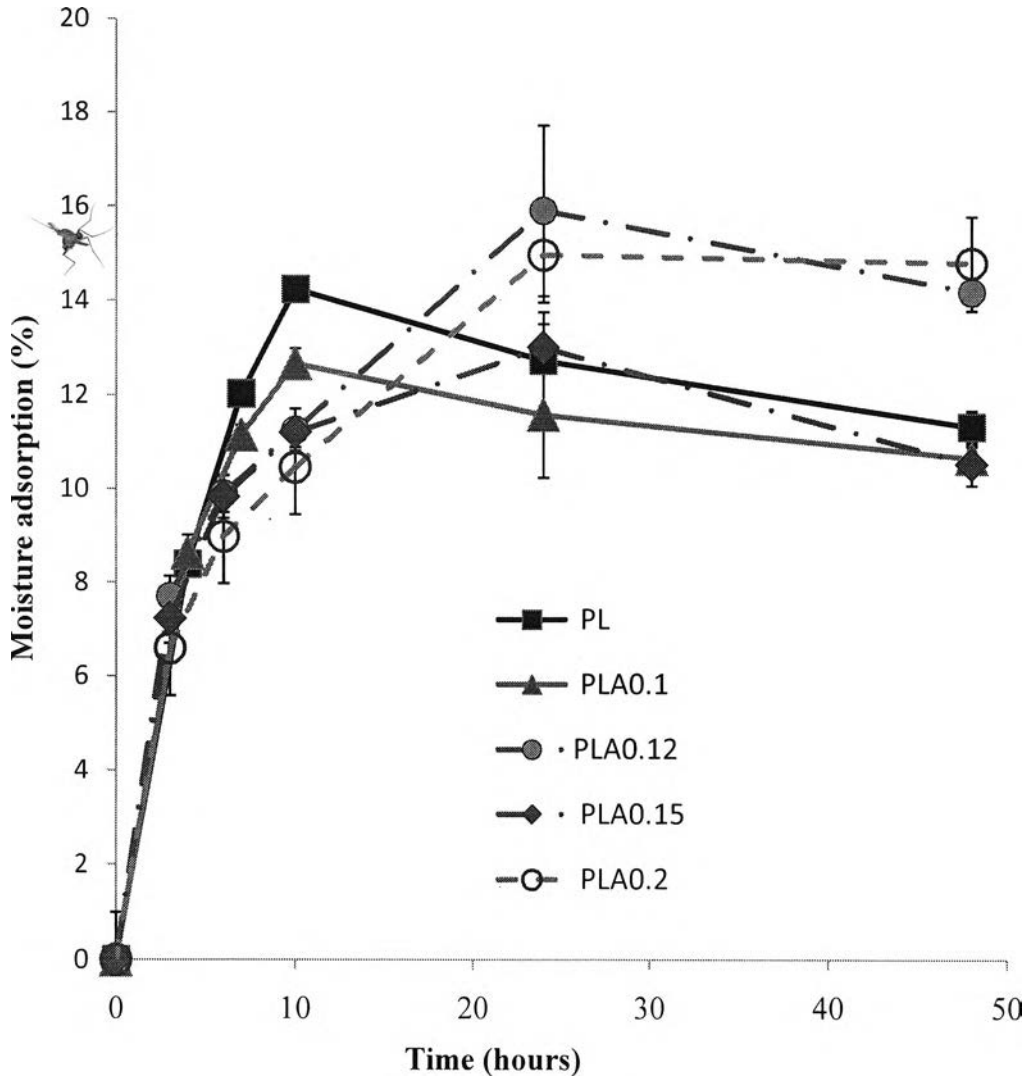


Figure 17 The effect of formulation parameters on the percentage of moisture adsorption (n=3)

PL : Pectic polysaccharide0.2% + Lactose 0.2%

PLA0.1: Pectic polysaccharide0.2% + Lactose 0.2%+Aerosil®0.1%

PLA0.12: Pectic polysaccharide0.2% + Lactose 0.2%+Aerosil®0.12%

PLA0.15: Pectic polysaccharide0.2% + Lactose 0.2%+Aerosil®0.15%

PLA0.2 : Pectic polysaccharide0.2% + Lactose 0.2%+Aerosil®0.2%

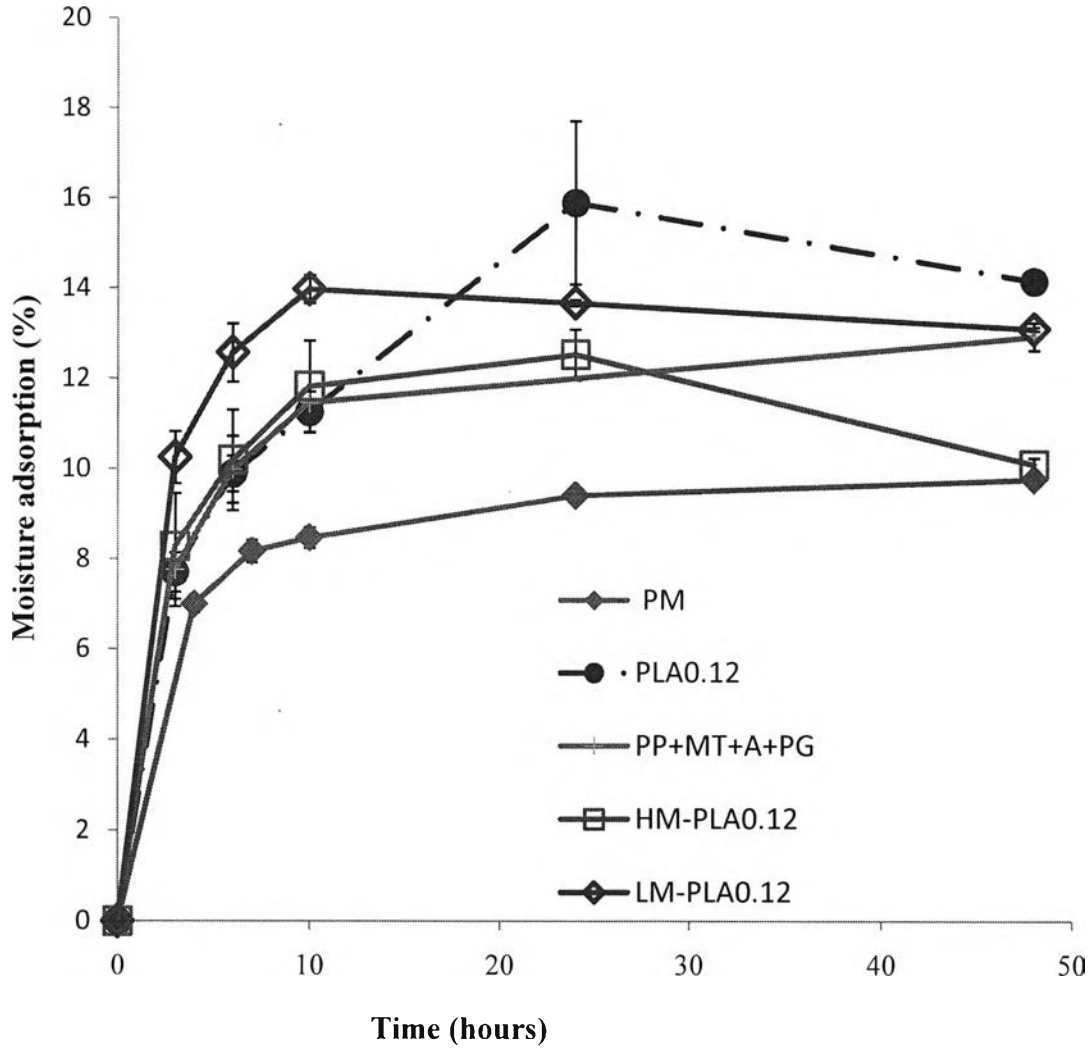


Figure 18 The effect of formulation parameters on the percentage of moisture adsorption (n=3)

PM : Pectic polysaccharide0.2% + Mannitol0.2%

PLA0.12: Pectic polysaccharide0.2% + Lactose 0.2%+Aerosil®0.12%

HM-PLA0.12: HM pectin0.2% + Lactose 0.2%+Aerosil®0.12%

LM-PLA0.12: LM pectin0.2% + Lactose 0.2%+Aerosil®0.12%

P+MT+A+PG: Pectic polysaccharide +maltodextrin+Aerosil+propylene glycol

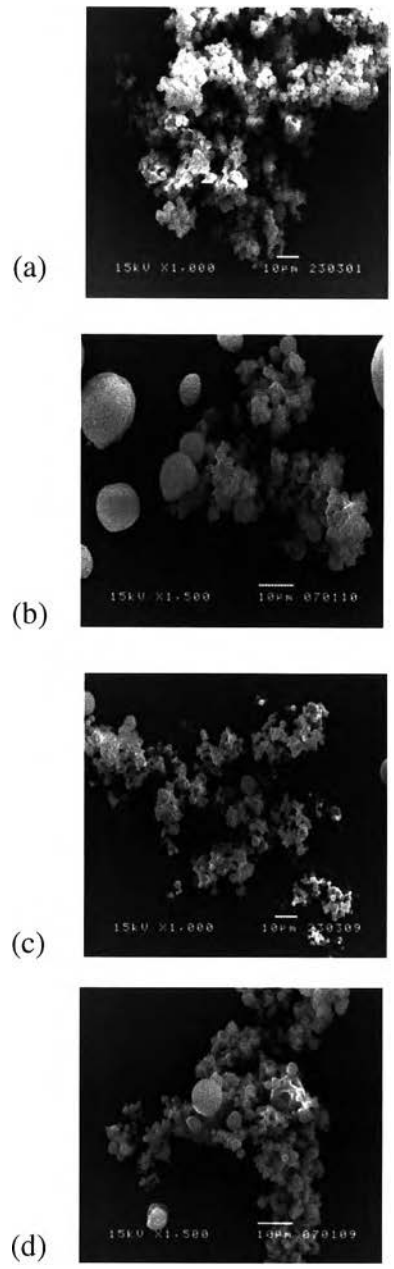


Figure 19 SEM photographs of pectic polysaccharide with maltodextrin, aerosil and propylene glycol microparticles exhibiting overall view. (a) 90-80-3 (b) 90-90-5 (c) 120-80-3 (d) 120-90-5 The numbers were inlet temperature(°C), aspirator rate (%), and feed rate (ml/min), respectively

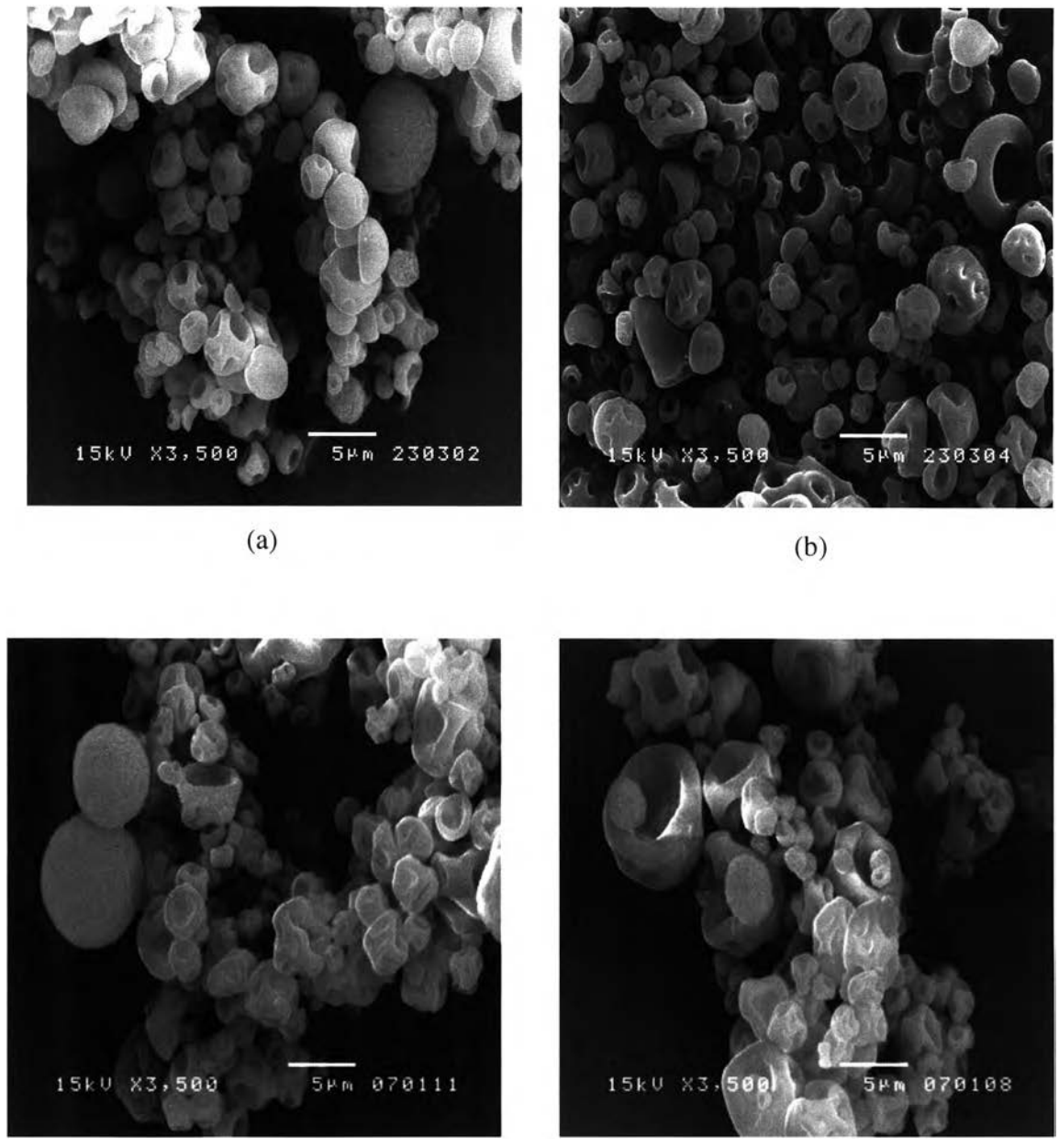


Figure 20 SEM photographs of pectic polysaccharide with maltodextrin, aerosil and propylene glycol microparticles exhibiting high enlargement .(a) 90-80-3 (b) 90-90-5 (c) 120-80-3 (d) 120-90-5 The numbers were inlet temperature(°C), aspirator rate (%), and feed rate (ml/min), respectively

The SEM photographs of the formulation of PP+MT+A+PG at various preparing conditions as shown in Figure 19-20 showed that microparticles were agglomerated together and presented external surfaces and some donut-shape at all processing conditions. The occurrence of concavities in the surface of particles would be associated to the rapid evaporation of the liquid droplet during the spray drying process (Favaro-Trindade et al.,2010). At low inlet temperature of 90°C microparticles had the narrow size distribution as shown in Figure 19(a-b) which was related to measurement by laser light scattering method. Whereas at the higher inlet temperature of 120 °C microparticles were more collapsed as shown in Figure 19(c-d). It may be due to impact of high drying rate of high temperature on morphology, increased particle wrinkling at elevated drying rate (Snyder,2008). The particle produced from the higher drying rate displayed a dimpled or first-order shell collapsed mode while the lower drying rate particles tended to be more spherical, with smaller surface wrinkles (Snyder,2008).

The detailed physics of the entire droplet to particle formation process is highly complex and dependent upon the coupled interplay between the process variables such as initial droplet size, feedstock concentration, and evaporation rate, along with the formulation physicochemical properties such as solubility, surface tension, viscosity and the solid mechanical properties of the forming particle shell (Snyder,2008). The SEM photographs of various formulations are presented in Figure 21. The morphology of the spray dried powder of pectic polysaccharide with maltodextrin, colloidal silica and propylene glycol as shown in Figure 21(a) had the most collapsed particles and the formulation of pectic polysaccharide with lactose and colloidal silica had donut- shape as shown in Figure 21(b) whereas the spray dried microparticles of HM pectin with lactose and colloidal silica (Figure 21c) and LM pectin with the same formulation (Figure 21 d) did not have donut-shape due to that commercial LM pectin had larger molecular weight than pectic polysaccharide leading to higher viscosity to maintain the stability of the droplets during spray-drying and commercial HM pectin had smaller impurity than pectic polysaccharide. The SEM photographs in the formulation of PP+MT+A+PG was different from the production reported by Harikarnpakdee et al. (Harikarnpakdee et

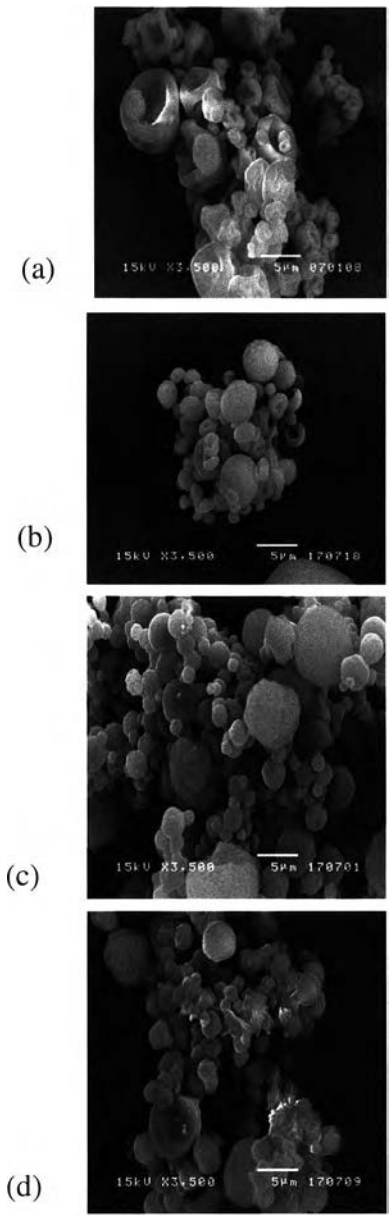


Figure 21 SEM photographs of spray dried microparticles from the preparing condition containing inlet temperature of 120 °C, 90% aspirator and feed rate of 5 ml/min with magnification of 3500x (a) The formulation of PP+MT+A+PG (b) The formulation of PLA0.12 (c) The formulation of HM –PLA0.12 (d) The formulation of LM-PLA0.12.

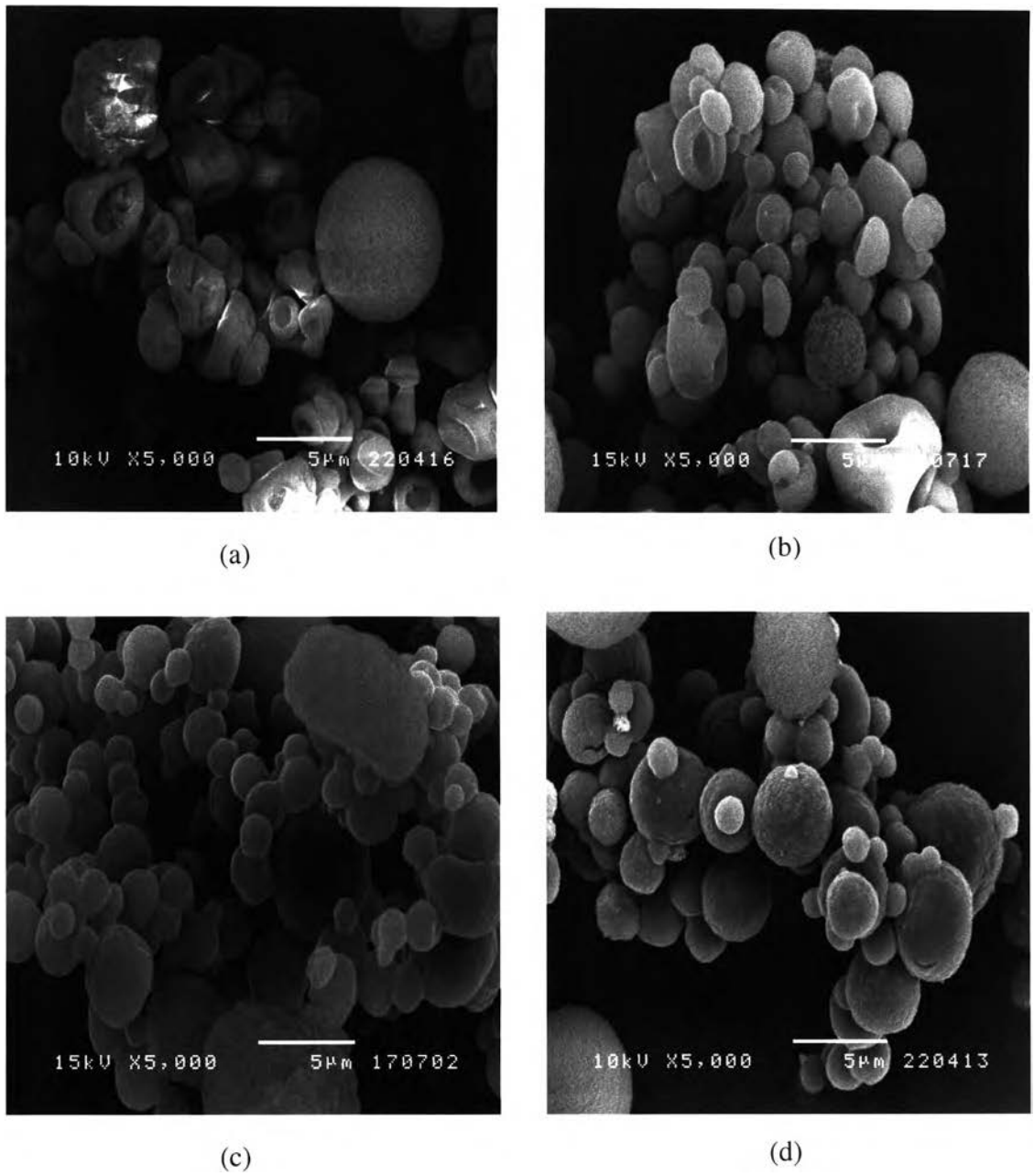


Figure 22 SEM photographs of spray dried microparticles from the preparing condition containing inlet temperature of 120 °C, 90% aspirator and feed rate of 5 ml/min with magnification of 5000 (a) The formulation of PP+MT+A+PG (b) The formulation of PLA0.12 (c) The formulation of HM –PLA0.12 (d) The formulation of LM-PLA0.12.

This results can be explained that shell formation will occur when one of the formulation components reaches its solubility and precipitates leading to the formation of a solid shell that may be either amorphous or crystalline. Low aqueous solubility components (which is colloidal silica in the formulation) tend to form corrugated particles so all formulations had not smooth surface (Snyder,2008).

4. Physical properties of BSA-loaded pectin microparticles

BSA was incorporated into pectin microparticles with the formulation of pectin, lactose, and Aerosil® 0.12%w/v at various loading levels. Physicochemical properties of the resultant microparticles are presented in Table 4.

Particle size of BSA–loaded spray dried powders ranged from 7.0 to 7.8 μm were significantly smaller than blank spray dried for both commercial pectins ranged from 10.95 to 13.99 μm ($p<0.05$: ANOVA) except BSA:HM pectin ratio of 1:10 which was not different from blank particle. However, for pectic polysaccharide BSA loading had no impact on particle size ($p>0.05$: ANOVA). And from the data of span ranged from 1.3 to 2.4 indicated that the size distribution of all obtained microparticles before and after loading was narrow.

Moisture content of BSA loaded spray dried microparticles seemed to be higher than blank spray dried powder except LM pectin as shown in Table 5 and Figure 23. These results were consistent with the report by Bosquillon et al. on the moisture content of spray dried powder with and without protein (Bosquillon et al.,2004). The spray dried pectic polysaccharide with/without BSA had higher moisture content than both of commercial pectin that may be due to the physical property of durian rinds that differ from property of citrus.

The swelling index of BSA loaded LM pectin microparticles (~54.76) and pectic polysaccharide (~45.92) tended to be lower than blank except at BSA: pectic polysaccharide ratio of 1:5. These results may be due to interaction of pectin and protein

reducing its carboxyl group to form hydrogen bonding with water (MacDougall et al.,2001). However, swelling property of HM pectin spray dried powder with loading of BSA much higher than blank high methoxylated pectin spray dried powder.

Mucoadhesion of all obtained BSA loaded spray dried microparticles were lower than blank spray dried microparticles. Pectin had hydroxyl groups and carboxyl groups to form hydrogen bond with functional groups in mucus layer. And it formed bond with protein at carboxyl groups. Lower mucoadhesive force of BSA loaded spray dried microparticles may be due to interaction of pectin and protein reducing its carboxyl group to form hydrogen bonding with functional groups in mucus layer (Liu et al.,2005).

Table 5 Physical properties of BSA-loaded pectin microparticles (n=3)

BSA:Pectin		Size (μm)	Span	Moisture content (%)	Swelling Index	Mucoadhesion (N)
PP-pectin	Blank	8.95 ± 0.30	1.15 ± 0.08	6.82 ± 0.08	45.92 ± 7.7	8.11 ± 0.75
	1:3	8.78 ± 0.16	1.20 ± 0.05	7.89 ± 0.07	34.52 ± 6.63	5.34 ± 1.13
	1:5	8.25 ± 0.29	1.61 ± 0.18	7.20 ± 0.14	65.83 ± 12.29	6.00 ± 0.49
	1:10	8.23 ± 0.72	1.60 ± 0.25	7.28 ± 0.22	41.07 ± 8.99	7.36 ± 1.36
HM-Pectin	Blank	10.95 ± 0.61	1.36 ± 0.10	5.00 ± 0.80	24.52 ± 4.31	10.44 ± 1.45
	1:3	7.07 ± 0.24	2.74 ± 0.41	6.51 ± 0.16	66.67 ± 0	5.70 ± 0.38
	1:5	7.34 ± 0.12	1.60 ± 0.09	5.79 ± 0.08	62.20 ± 5.18	7.74 ± 1.88
	1:10	11.99 ± 0.69	1.58 ± 0.08	5.69 ± 0.30	97.50 ± 26.86	8.21 ± 0.63
LM-Pectin	Blank	13.98 ± 1.03	1.85 ± 0.07	6.23 ± 0.10	54.76 ± 4.12	14.45 ± 1.12
	1:3	7.40 ± 0.34	1.53 ± 0.27	6.47 ± 0.06	35.71 ± 12.37	6.33 ± 0.79
	1:5	7.26 ± 0.17	1.93 ± 0.01	5.52 ± 0.27	35.04 ± 2.96	7.39 ± 2.21
	1:10	7.77 ± 0.13	2.04 ± 0.07	6.04 ± 0.00	42.06 ± 8.36	11.95 ± 0.64

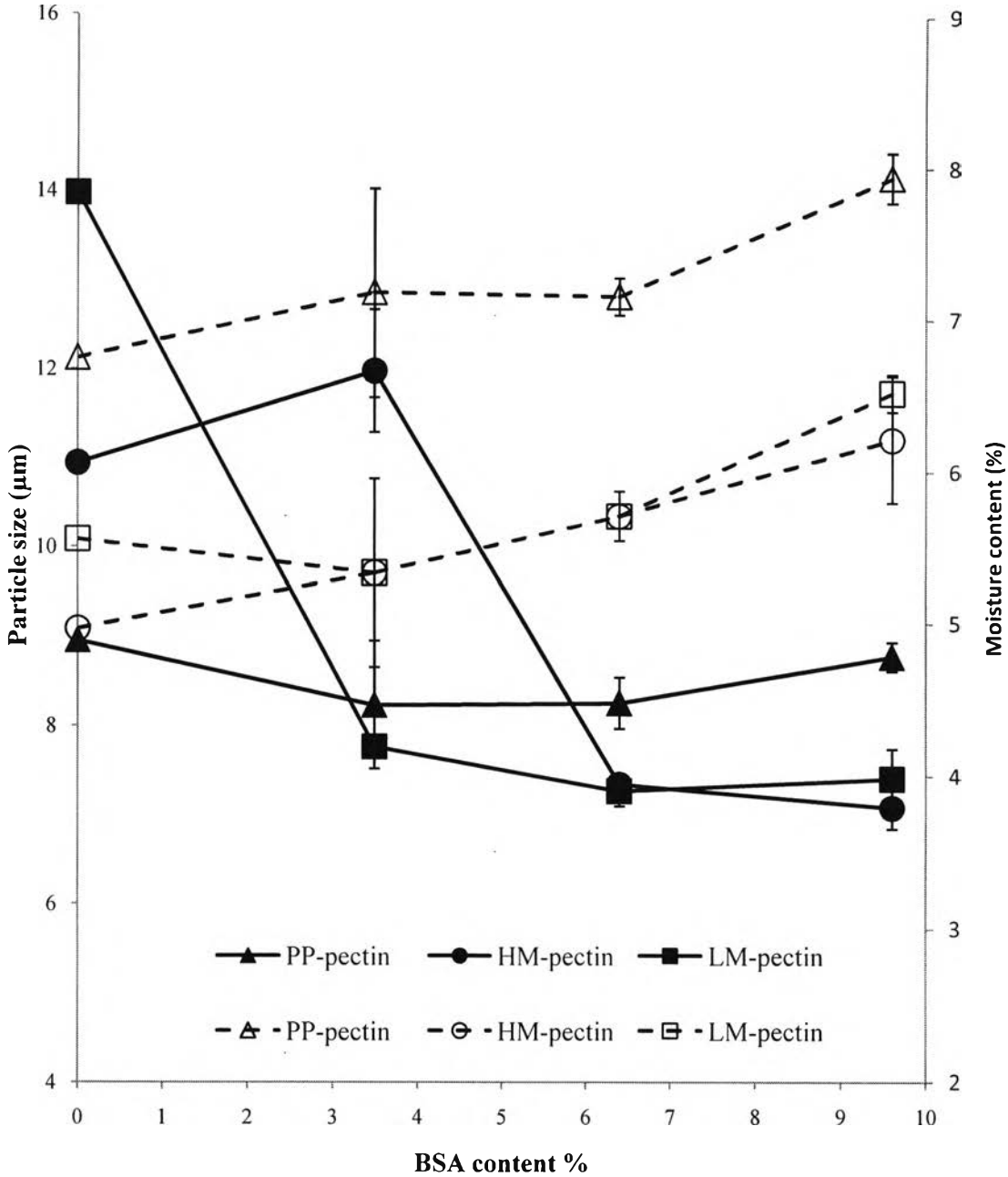


Figure 23 Particle size and moisture adsorption of BSA loaded pectin microparticles. The dashed line (---)was percentage of moisture content and the solid line (—) was particle size (n=3)

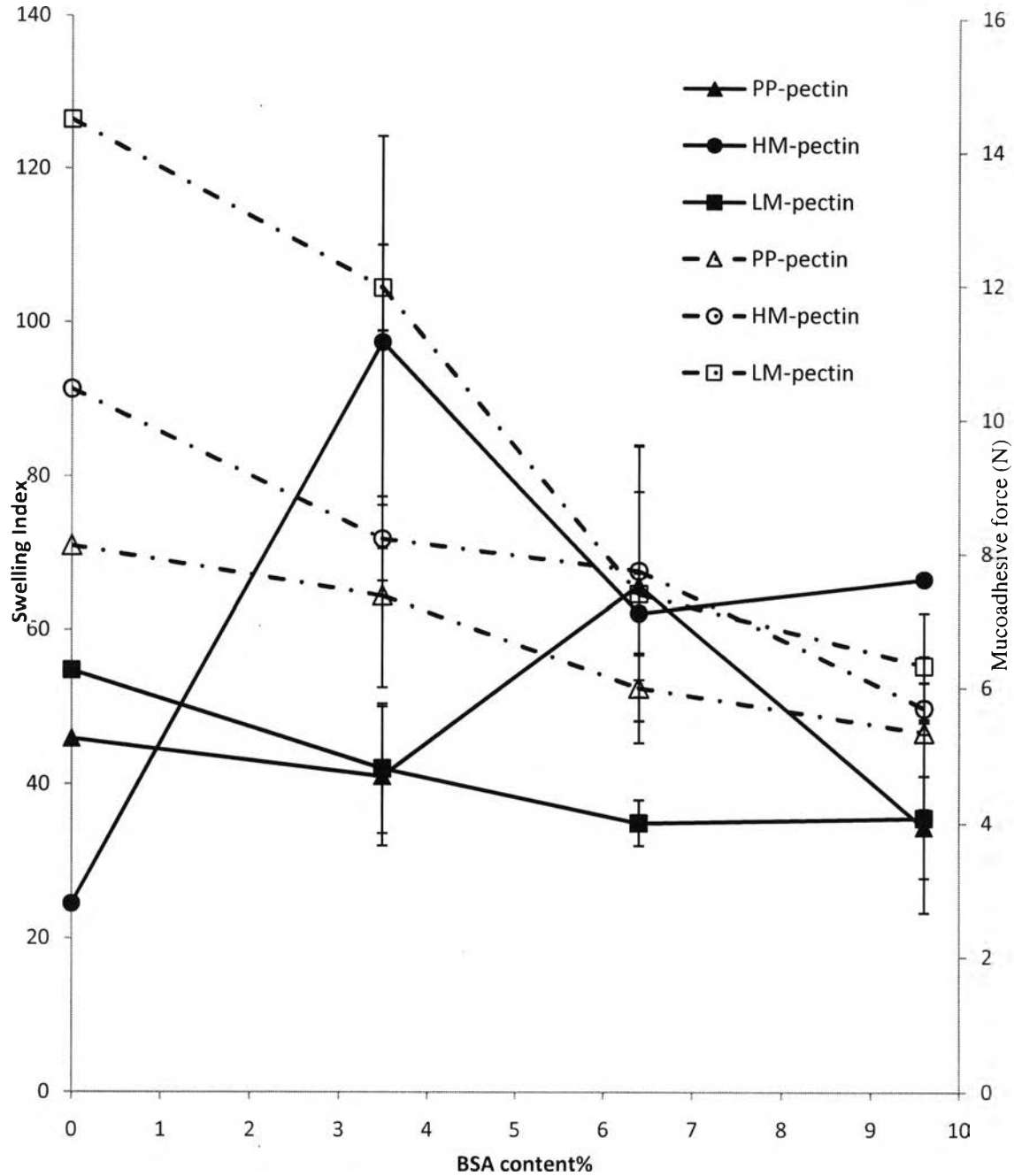


Figure 24 Mucoadhesive force and swelling index of BSA loaded pectin microparticles. The dashed line (---)was mucoadhesive force and the solid line (—) was swelling index. (n=3)

The SEM photographs revealed the spherical particle shape of spray dried blank pectin at all types of pectin as shown in Figure 25 -30. BSA loaded into three types of pectin microparticles slightly affected the smoothness of the microparticles which was ranked 1:10 > 1:5 > 1:3. These different external structures could be attributed to the nature of the surface crust. The addition of protein may increase the porosity but may also change the flexibility of the surface, mechanical strength, evaporation of water linked to protein (Yoshii et al.,2008). Spray dried blank microparticles of all types of pectin seemed to be more aggregated than BSA loaded pectin microparticles. The SEM photographs (Figure 25-26) showed that the spray dried pectic polysaccharide were donut-shape whereas both commercial pectin seemed to be round shape and the size distribution of BSA loaded LM pectin (Figure 29 – 30) at BSA:pectin ratio of 1:10 was broad which was related to data from Table 4.

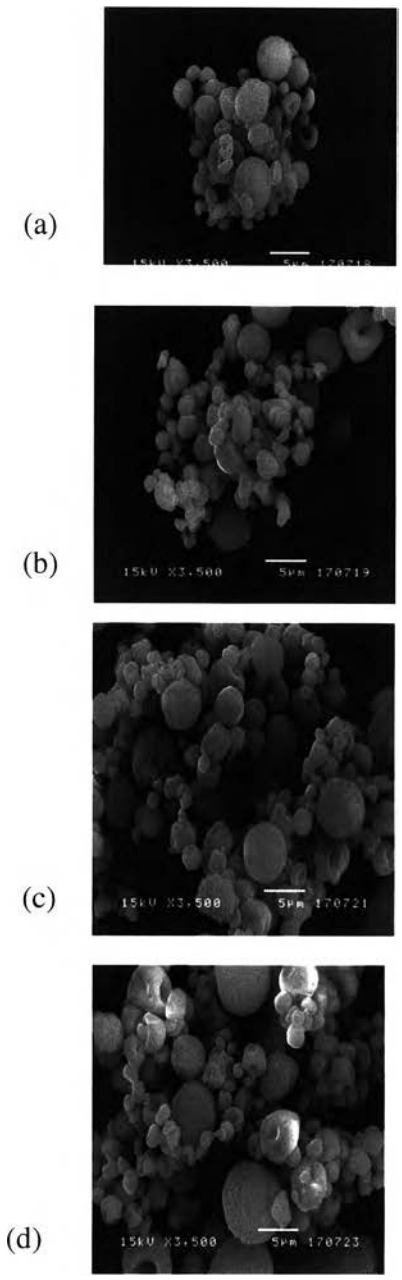
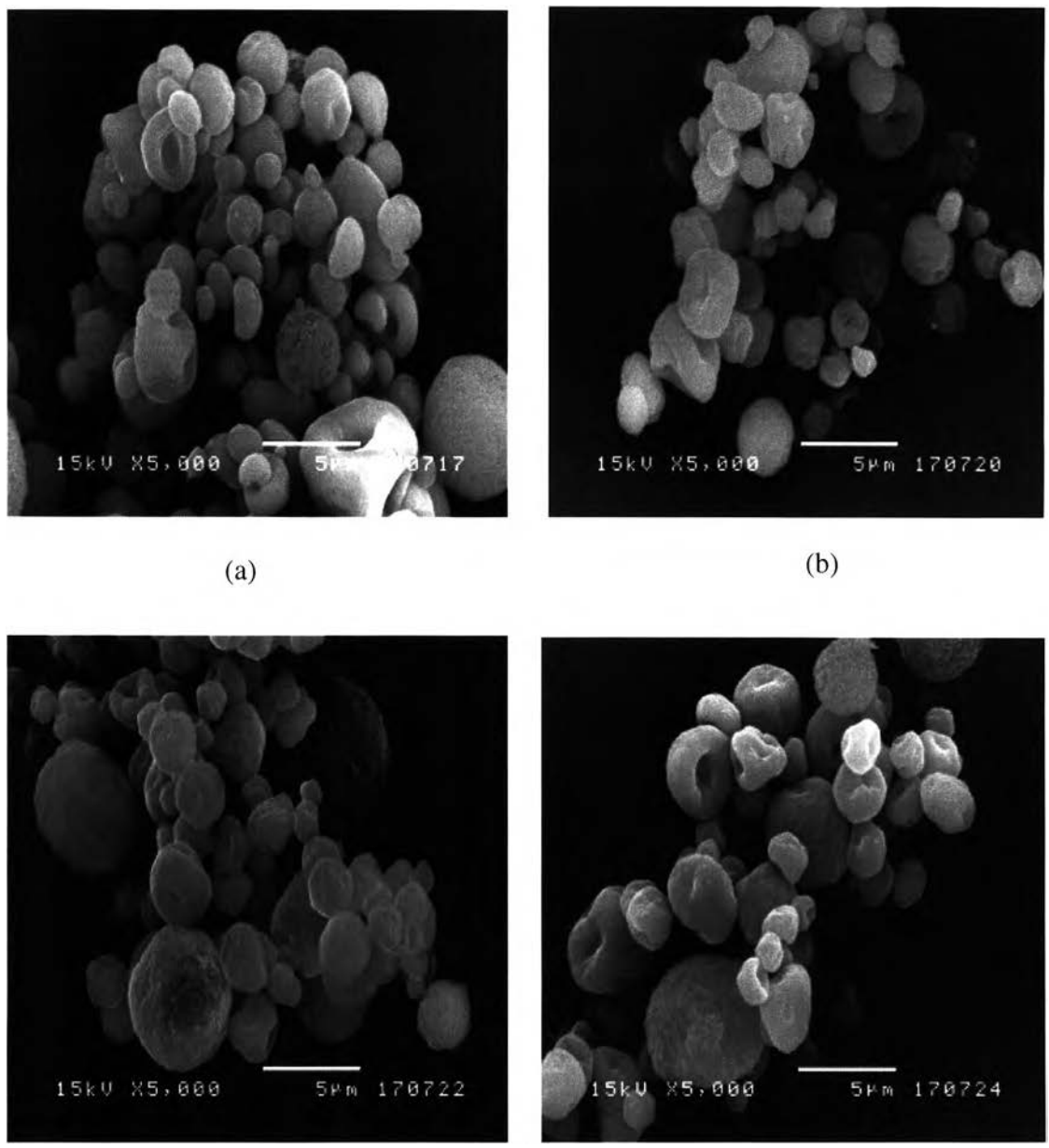


Figure 25 SEM photographs of various BSA:pectic polysaccharide weight ratio compared to blank with magnification of 3500. (a)blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10



(a) (b)

(c) (d)

Figure 26 SEM photographs of various BSA:pectic polysaccharide weight ratio compared to blank with magnification of 5000. (a)blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10

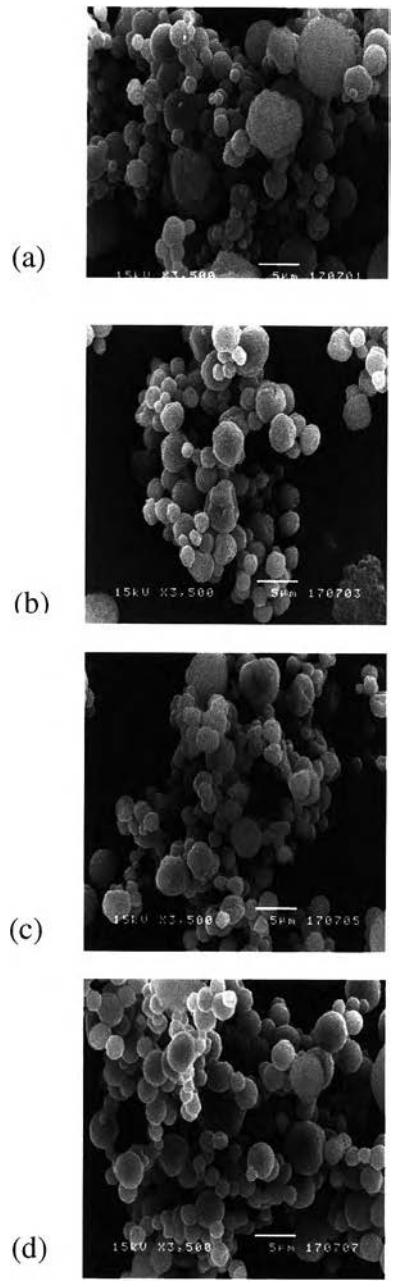


Figure 27 SEM photographs of various BSA:HM pectin weight ratio compared to blank with magnification of 3500. (a)blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10

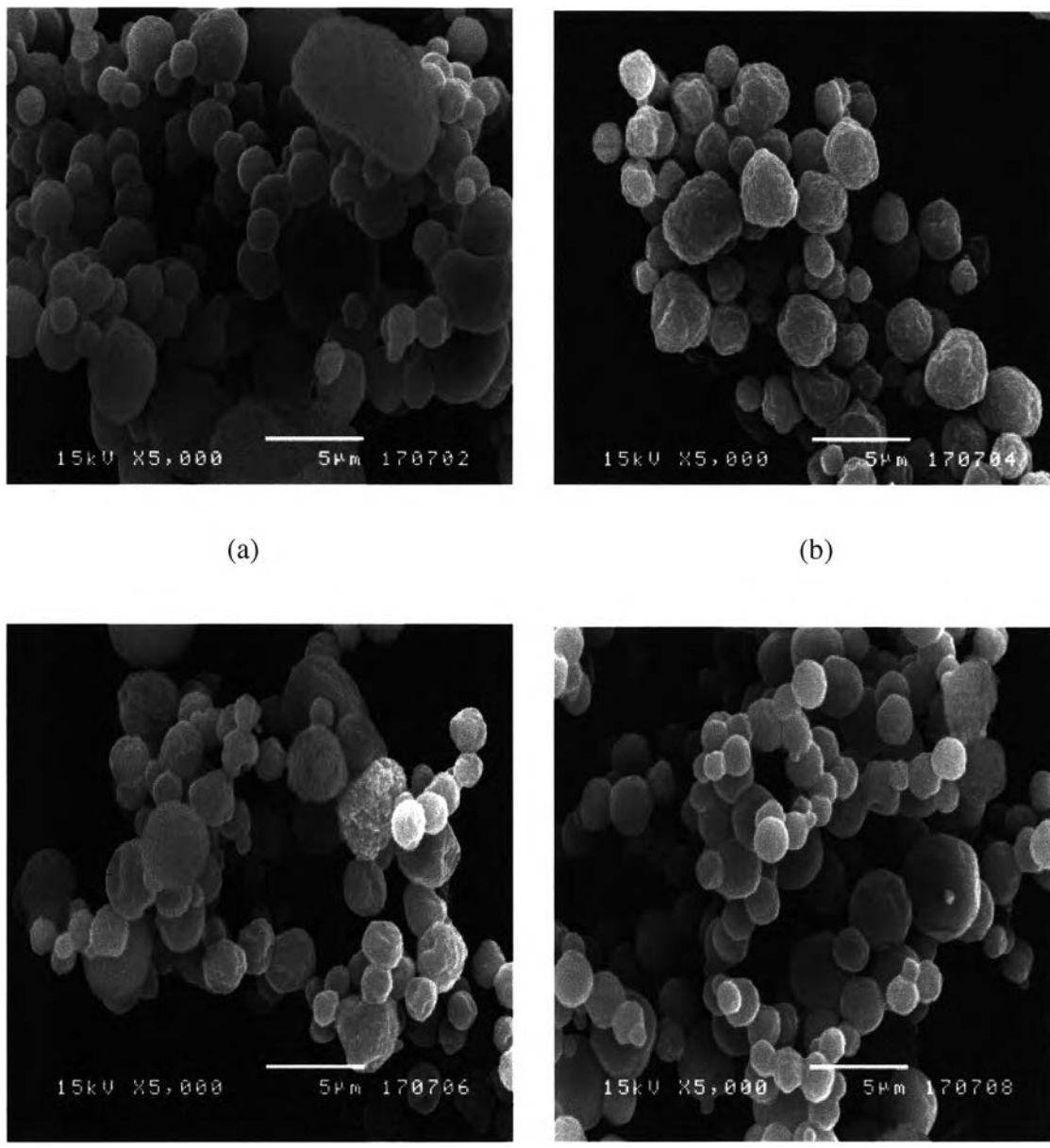


Figure 28 SEM photographs of various BSA:HM pectin weight ratio compared to blank with magnify of 5000. (a) blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10

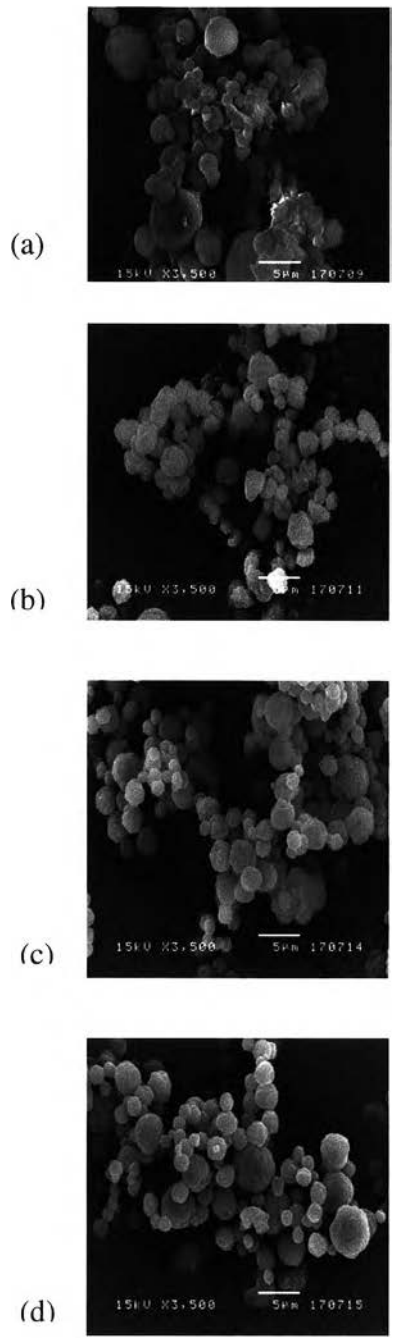


Figure 29 SEM photographs of various BSA:LM pectin weight ratio compared to blank with magnification of 3500. (a)blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10

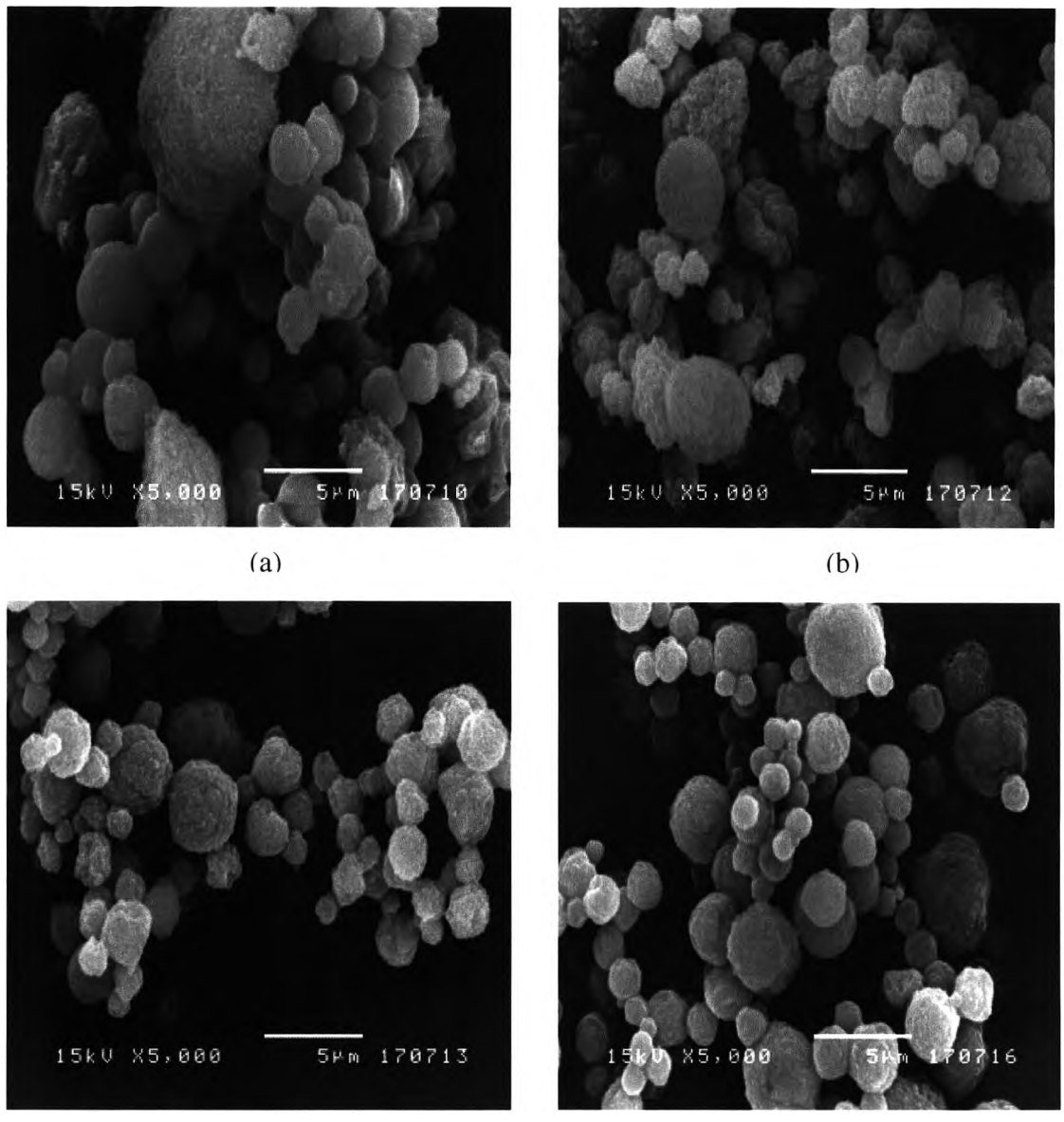


Figure 30 SEM photographs of various BSA:LM pectin weight ratio compared to blank with magnification of 5000. (a) blank spray dried pectin (b) BSA:pectin = 1:3 (c) BSA:pectin = 1:5 (d) BSA:pectin = 1:10

5. Chemical properties of BSA-loaded pectin microparticles

5.1. Infared absorption study

Pectin has free carboxyl group that imparts negative charge to these molecules. Protein molecule such as bovine serum albumin has positive charge at acidic pH due to presence of amino groups. During mixing to be feedstock solution and spray drying carboxyl groups in pectin may interact with amino groups in protein to form a complex that contain amide. FT-IR analysis was carried out to confirm formation of amide due to interaction of free carboxyl and amino group present in pectin and protein, respectively.

FTIR spectra of unprocessed BSA and BSA loaded pectin microparticle at the ratio of 1:1 was obtained. Figure 31 shows the spectra of unprocessed BSA, unprocessed three types of pectin, and BSA loaded three types of pectin microparticles. The FTIR spectra of three types of pectin depict the broad, strong area of absorption between 3600 and 2500 cm^{-1} refers to O-H stretching absorption due to inter- and intramolecular hydrogen bonds (Gnanasambandam et al.,2000). In the case of esterified pectins, an $O\text{-}CH_3$ stretching band would be expected between 2950 and 2750 cm^{-1} due to methyl esters of galacturonic acid. However, due to a large O-H stretching response occurring in a broad region (3600-2500 cm^{-1}), the $O\text{-}CH_3$ activity is masked and not a reliable indicator of methoxylation. Stronger bands occurring between 1760 – 1745 cm^{-1} and between 1640 – 1620 cm^{-1} indicate the ester carbonyl (C=O) groups and carboxylate ion stretching band (COO-), respectively (Gnanasambandam et al.,2000). It was observed that the ester carbonyl group band increased in intensity and band area for high methoxylate pectin of pectic polysaccharide more than HM pectin but did not appeared in LM pectin (Figure 31 F , G). Thus it was reasonable to conclude that pectic polysaccharide was a high methoxylate pectin which had higher degree of esterification than this commercial HM pectin.

FTIR spectra of pectin (Figure 31 A, B, C) showed peak of free carboxylate group at 1640, 1643 and 1642 for PP, HM and LM pectin, respectively, which were disappeared in BSA loaded pectin microparticles (Figure 31 E, F, G). A characteristic peak for amide in the region of 1500 – 1650 cm^{-1} increased in intensity and shifted in the BSA loaded pectin microparticles from 1532 of unprocessed BSA to 1538, 1539 and 1541 of PP, HM, LM pectin, respectively. These results were able to confirm formation of complex due to reaction between amino group of BSA and carboxylic group of pectin (Saravanan et al.,2010). In addition, the FTIR spectra for ester carbonyl group of BSA loaded HM pectin and BSA loaded pectic polysaccharide (Figure 31 E,F) appeared to slightly shift from 1763 to 1754 and from 1760 to 1751, respectively.

The FTIR analysis can be used to evaluate stability of protein by comparing to unprocessed protein. The peptide group, the structural repeat unit of proteins, give up to 9 characteristic bands named amide A, B, I,II,...,VII. Amide I and II bands are two major bands of the protein infared spectrum. The amide I band (1600 – 1700 cm^{-1}), which is the sum of overlapping second structure component bands, is mainly associated with the C=O stretching vibration (70 –85%) and is directly related to backbone conformation. Amide II results from the N-H bending vibration (40-60%) and from the C-N stretching vibration (18-40%). This band is conformational sensitive (Barth,2007). The amide I spectra of unprocessed BSA resulted in a strong band at 1661 cm^{-1} (Figure 29 D) which was similar to spectra of BSA from PP, HM, LM pectin microparticles (1661, 1663, and 1662 cm^{-1}). For amide II spectra of unprocessed BSA resulted in a band at 1550 cm^{-1}. Amide II spectra of BSA from all types of PP, HM and LM pectin increased in intensity and shifted from 1532 to 1538, 1539 and 1541, respectively. Secondary structure analysis of protein is nearly exclusively done using the amide I band, but amide II band as well as the near-infared region have also been shown to be useful. Therefore, it was concluded that the spray drying process at this condition did not affect the principal secondary structure of BSA.

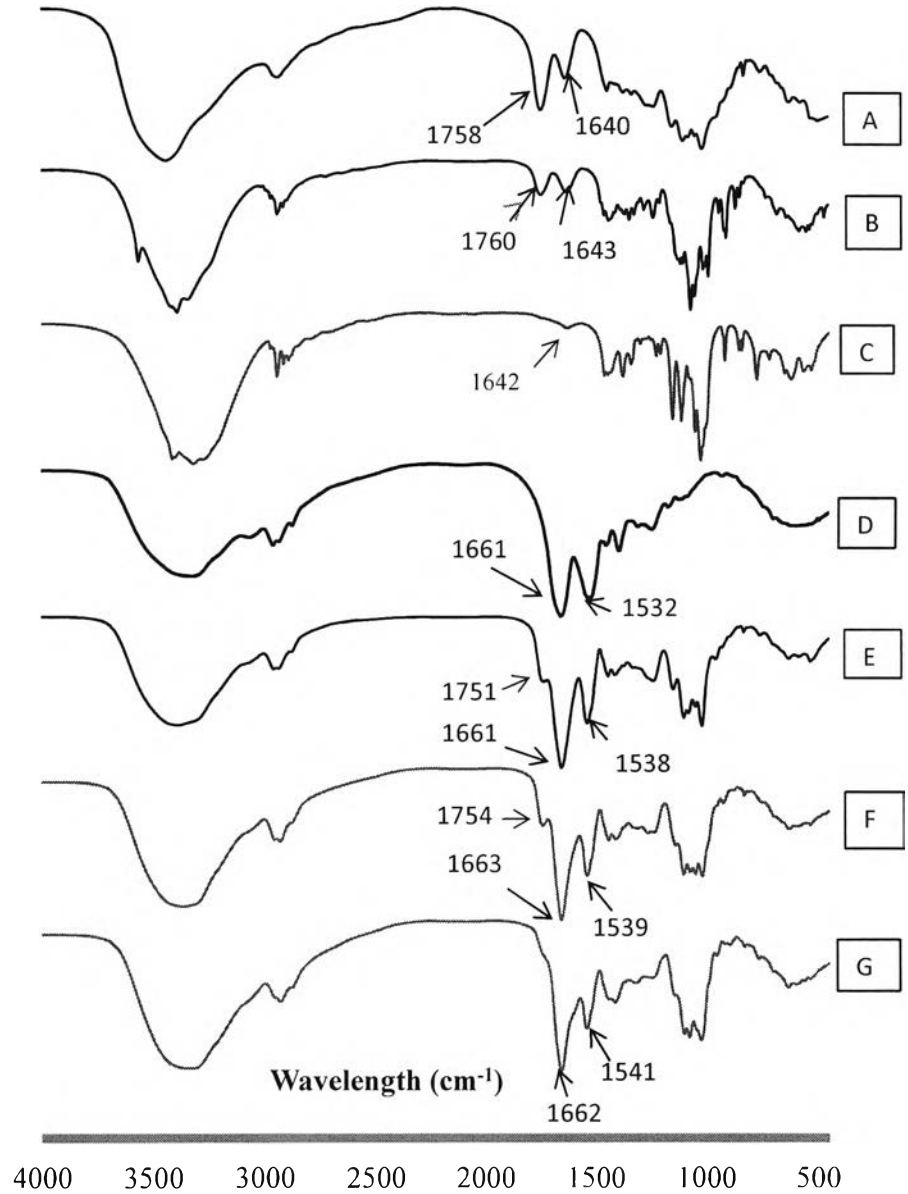


Figure 31 FT-IR Spectra of pectin, unprocessed BSA and BSA loaded pectin microparticle : (A) PP, (B) HM pectin, (C) LM pectin, (D) unprocessed BSA, (E) BSA loaded PP, (F) BSA-loaded HM pectin (G) BSA-loaded LM pectin

5.2. Protein content determination

Many polymers were used to entrap protein. In this study, three types of pectin were compared. It was found that pectic polysaccharide (PP) showed the higher protein content than those of LM pectin and HM pectin at the BSA:pectin weight ratio of 1:5 and 1:10 as shown in table 6 that may be due to surface composition of protein. At BSA:pectin weight ratio of 1:3, the protein loading of all types of pectin were not different significantly ($p>0.05$: ANOVA). Amount of pectin were decreased proportionally to protein increasing in order to be an equal solid content on each formulation. Thus the more pectin were incorporated (lower BSA:pectin ratio),the lower was the protein loading in the microparticles. Consequently, different BSA:pectin ratio had different protein loading.

Table 6 The percentage protein content on three types of pectin

BSA: Pectin	Theory	PP pectin*	LM pectin*	HM pectin*
1:3	9.60	8.21 ± 7.81	8.66 ± 0.80	9.34 ± 1.17
1:5	6.40	9.22 ± 0.55	5.17 ± 0.64	5.46 ± 0.56
1:10	3.49	7.46 ± 0.71	3.57 ± 0.30	3.71 ± 0.29

*n=9

5.3. *In vitro* protein release

The release of BSA from the microparticle obtained under three types of pectin and various protein polymer ratio was investigated (Figure 32). Different types of pectin produced different drug release patterns. The initial burst release was approximately 80% for all ration of BSA-loaded pectic polysaccharide due to the small particle size. The particle size is a very important characteristic for spray-dried products, in particular, polymer degradation and release of the encapsulated product (Prinn et al.,2002). In addition, the initial burst release of BSA from LM pectin was lower than from HM pectin microparticle due to that the higher molecular weight of LM pectin formed the thicker gel. BSA released from all obtained microparticle were ranged PP > HM>LM due to the highest molecular weight of LM pectin. This may be due to the fact that larger molecule formed higher viscosity gel after hydrating by water. The release of drug from the polymer microparticles was controlled by the formation of a gel which slowed diffusion of the drug across the viscous boundary layer close to the dissolving surface and:or slowed crystallization of the amorphous drug (lower solubility) as a result of supersaturation in the boundary layer (Alhalaweh et al.,2009).

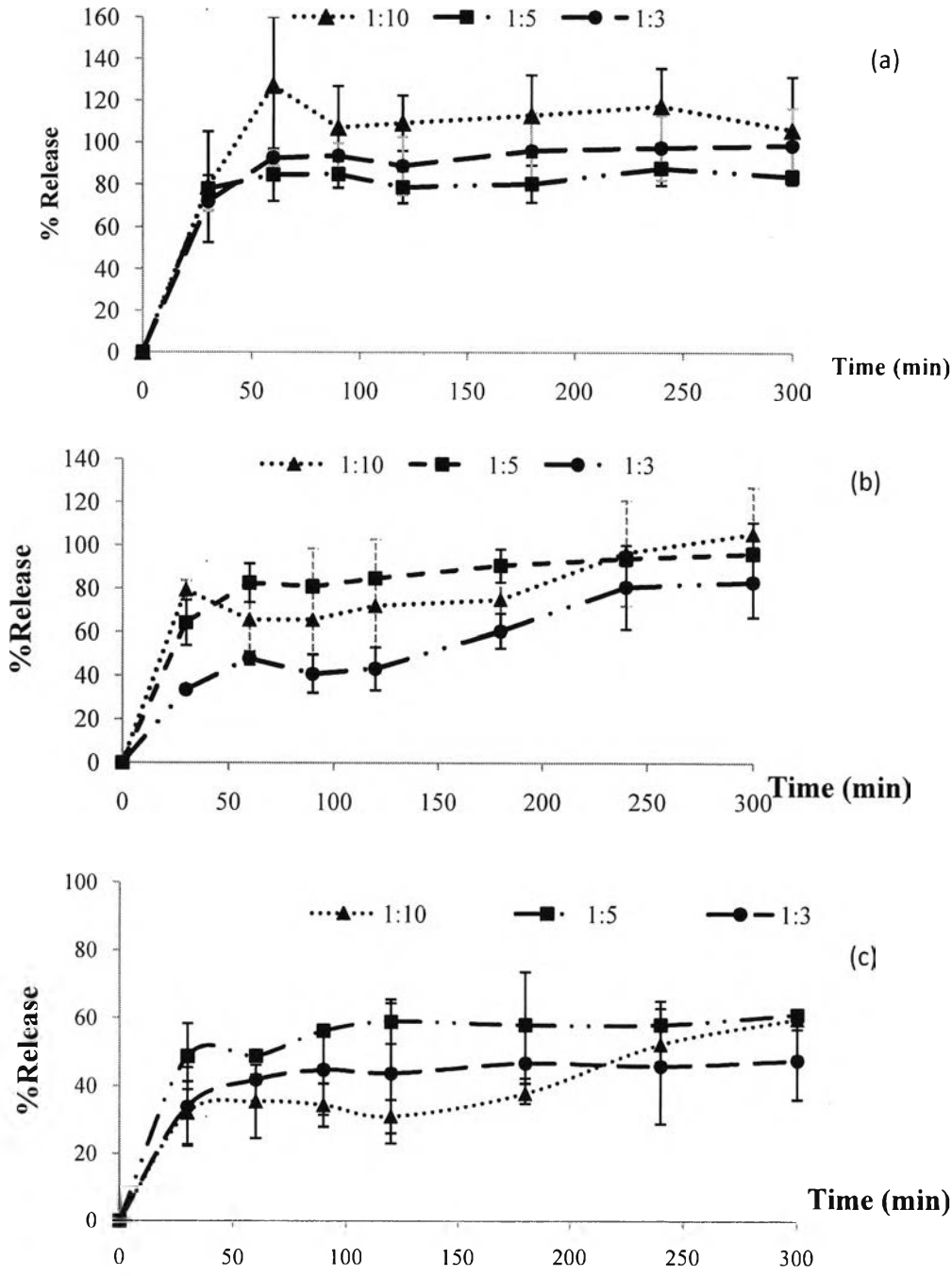


Figure 32 Release profiles of BSA from (a) PP microparticle, (b) HM pectin microparticle, (c) LM pectin microparticle in 0.2% SLS in water

5.4. Protein properties

One of the most important consideration for protein loading into pectin microparticles was its structural stability after preparing. Many preparing processes were developed to entrap drugs in polymer such as spray freeze drying (Zijlstra et al.,2009), coacervation (de Kruif et al.,2004), spray drying (Benchabane et al.,2007) but some processing conditions may affect the stability of protein. Therefore, protein integrity was considered necessary to be evaluated after preparing process. In this study, BSA molecule was exposed to the drastic conditions in preparation process such as high temperature, high pressure and mechanical force. These conditions might affect irreversible denaturation and loss of protein activity. Thus, protein structure and its integrity needed to be evaluated after preparing. The technique was used to compare the conformational change between native and modified protein was circular dichroism.

The circular dichroism was used to observe secondary and tertiary structures of protein release from preparations. Protein secondary structure can be determined by CD spectroscopy in the 'far-UV' spectral region (190-250 nm), whereas the 'near-UV' spectral region (250-320 nm) can be sensitive to certain aspects of tertiary structure by using 1 ml of 1mg/ml of protein (Ranjbar et al.,2009). Nevertheless, in this study, the tertiary could not be noticed because of low concentration of protein in the solution. Therefore, only the CD spectra of BSA secondary structures after spray drying process presented in Figure 31 are 'w'-shaped spectra with trough around 222 and 208 being indicative of the presence of α helical structures (Ranjbar et al.,2009) similar to the CD spectra of BSA microparticles by co-axial electrospray (Xie et al.,2008). The relationship between ellipticity and wavelength showed the same pattern in different BSA concentrations from microparticles. The calculated molar ellipticity showed that there was a little change in intensity of CD spectra of BSA released from HM pectin microparticles at the BSA:pectin ratio of 1:5 and LM pectin at the BSA:pectin ratio of 1:3 and 1:10 compared to unprocessed BSA as shown in Figure 33. These results may be due to higher concentration of released BSA but no change in secondary structure. It could be concluded that the selected condition of spray drying process was gentle to prepare

protein loaded pectin microparticles without any destructive effect on the protein structure. The spray drying of antibody had been reported that IgG displayed the CD spectra in β sheet structure and similar to pure antibody solution (Schüle et al.,2007).

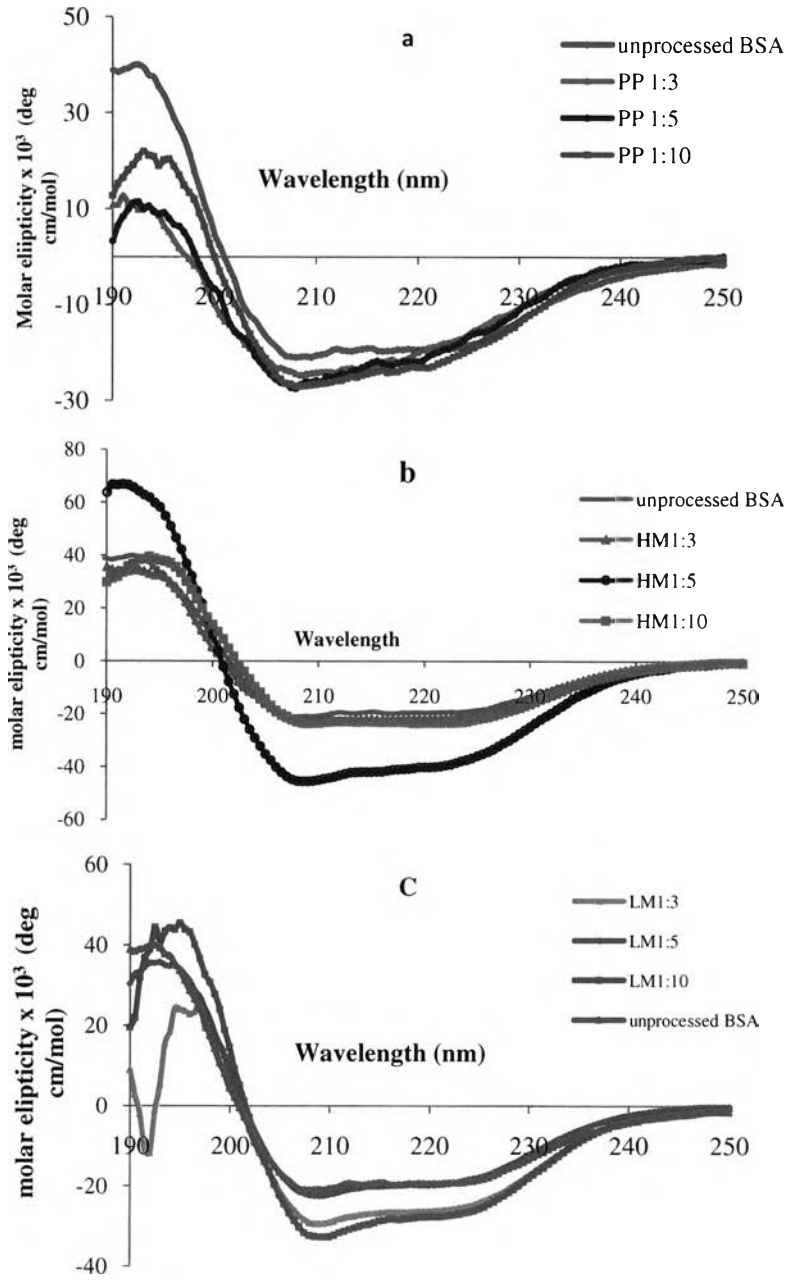


Figure 33 CD spectra of BSA-loaded pectin microparticles from three types of pectin at BSA : polymer ratio of 1:3, 1:5 and 1:10 (A) PP (B) HM pectin (C) LM pectin

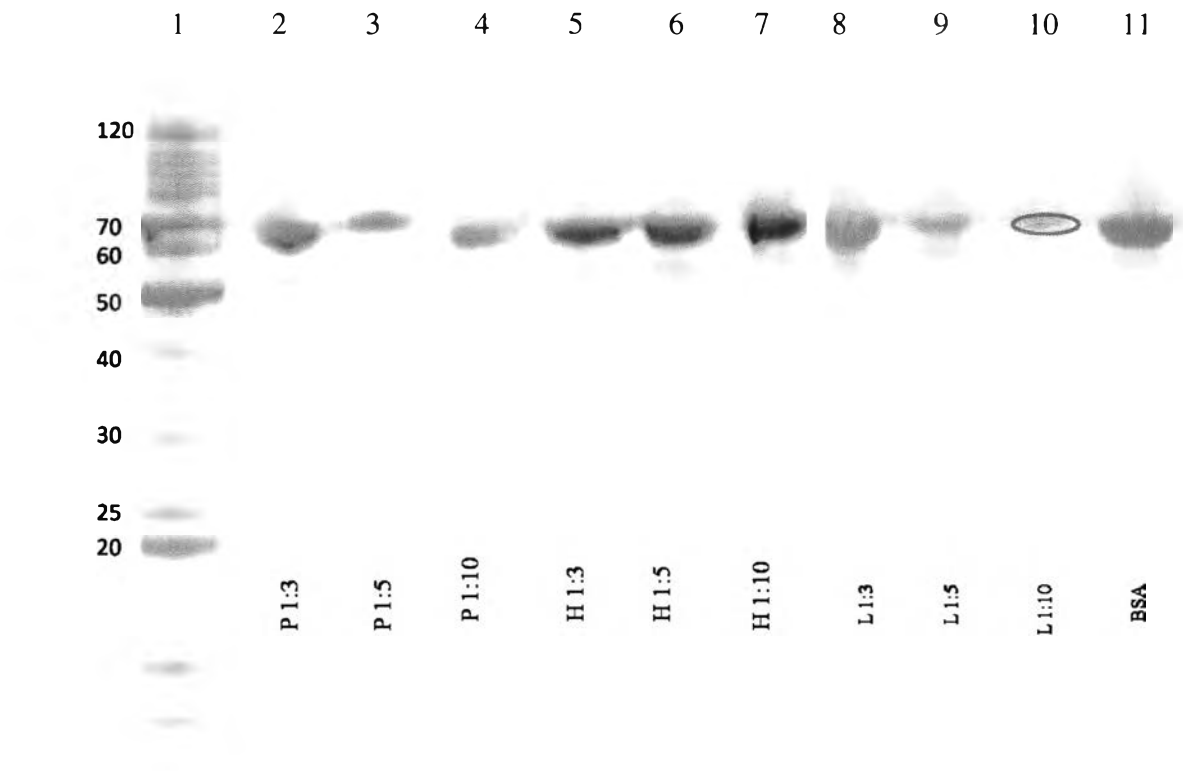


Figure 34 SDS-PAGE bands of BSA from Lane (1): molecular weight standards broad range; lane (2): PP 1:3; lane (3): PP 1:5; lane (4): PP 1:10, lane (5); HM 1:3 lane (6): HM 1:5; lane(7): HM1:10; lane (8): LM 1:3; lane (9): LM 1:5; lane (10): LM 1:10; lane (11): BSA raw material.

The SDS-PAGE technique was also used to confirm protein structure after preparing. The different protein molecular size could be separated in different moving rate in polyacrylamide gel, lower size showed more rapid diffusion than higher size. Figure 34 illustrates the SDS-PAGE molecular weight standards broad range (Invitrogen, USA) at lane 1. The molecular weight of bovine serum albumin is 66.4 KDa (Determan et al.,2004) and is represented in lane 11. Gel electrophoresis analysis (SDS-PAGE) of spray dried samples (Figure 34) identified that the BSA appeared to remain monomeric, with having appeared as a single band of 66 KDa for all types of pectin and all BSA concentration, this indicates that the primary structure of BSA loaded pectin microparticles by this spray drying condition was generally conserved.

microparticles by this spray drying condition was generally conserved.

6. Biopharmaceutical properties of BSA-loaded pectin microparticles

6.1 Cytotoxicity study

The cytotoxicity of three different type of pectin on the human nasal epithelial cells was investigated by MTT assay, an indicator of cellular mitochondrial dehydrogenase activity. MTT [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a tetrazolium salt that is oxidized by mitochondrial dehydrogenase in living cells to gives dark blue formazan product. Damaged or dead cells show reduced or no dehydrogenase activity. Thus, the optical density of the cell lysate after MTT treatment is linearly correlated with the dehydrogenase activity, and also reflects the cell viability. The changes of cell viability with the incubation of various concentrations of microparticles (0.5, 1.0, 1.5 mg/ml), pectin type and various protein:polymer ratios for 48 hours were depicted in Figure 35.

Typically, the percentage of cell viability as shown in Figure 35 was significantly increased as the particle loading was decreased due to less quantity of substance which may be toxic and at the formulation of pectic polysaccharide with colloidal silica at all ratios of BSA:pectin ($p<0.05$: ANOVA). For the formulation of LM pectin, HM pectin and PP pectin without colloidal silica, the cell viability was not different significantly at all ratios of BSA:pectin. It was found that all obtained BSA-loaded pectin microparticles were relatively non-toxic to nasal cell. The lowest percentage of cell viability was perceived with BSA-loaded pectic polysaccharide (PP) microparticle containing colloidal silicon dioxide at the highest particle loading ($p<0.05$: ANOVA). This contrast to the formulation without colloidal silica. It was concluded that Aerosil® affected cell viability in case of spray drying with PP although microparticles of both commercial pectins had also Aerosil®.

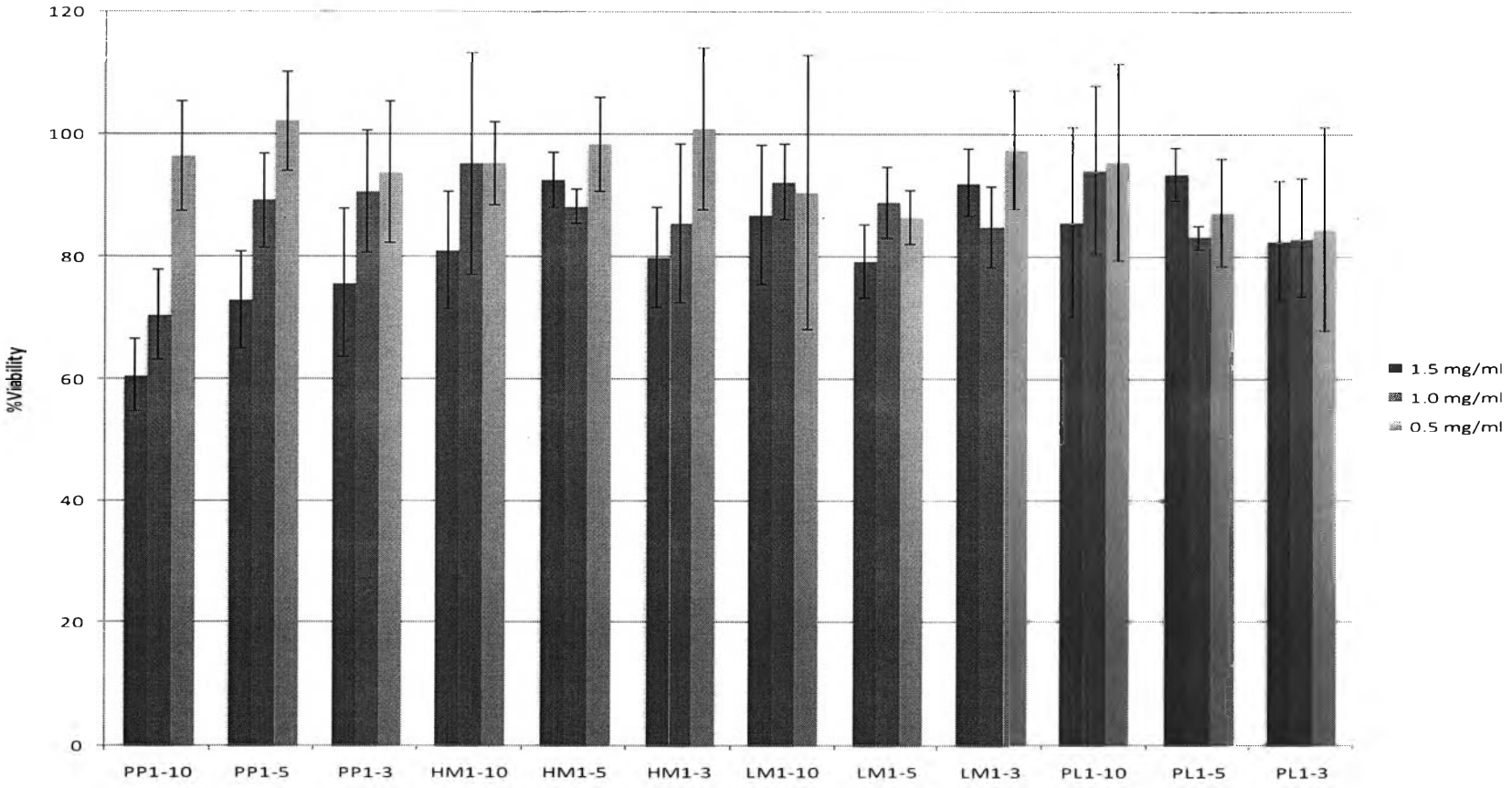


Figure 35 Percentage of nasal cell viability, co-incubated with BSA loaded pectin microparticles as a function of microparticle loading

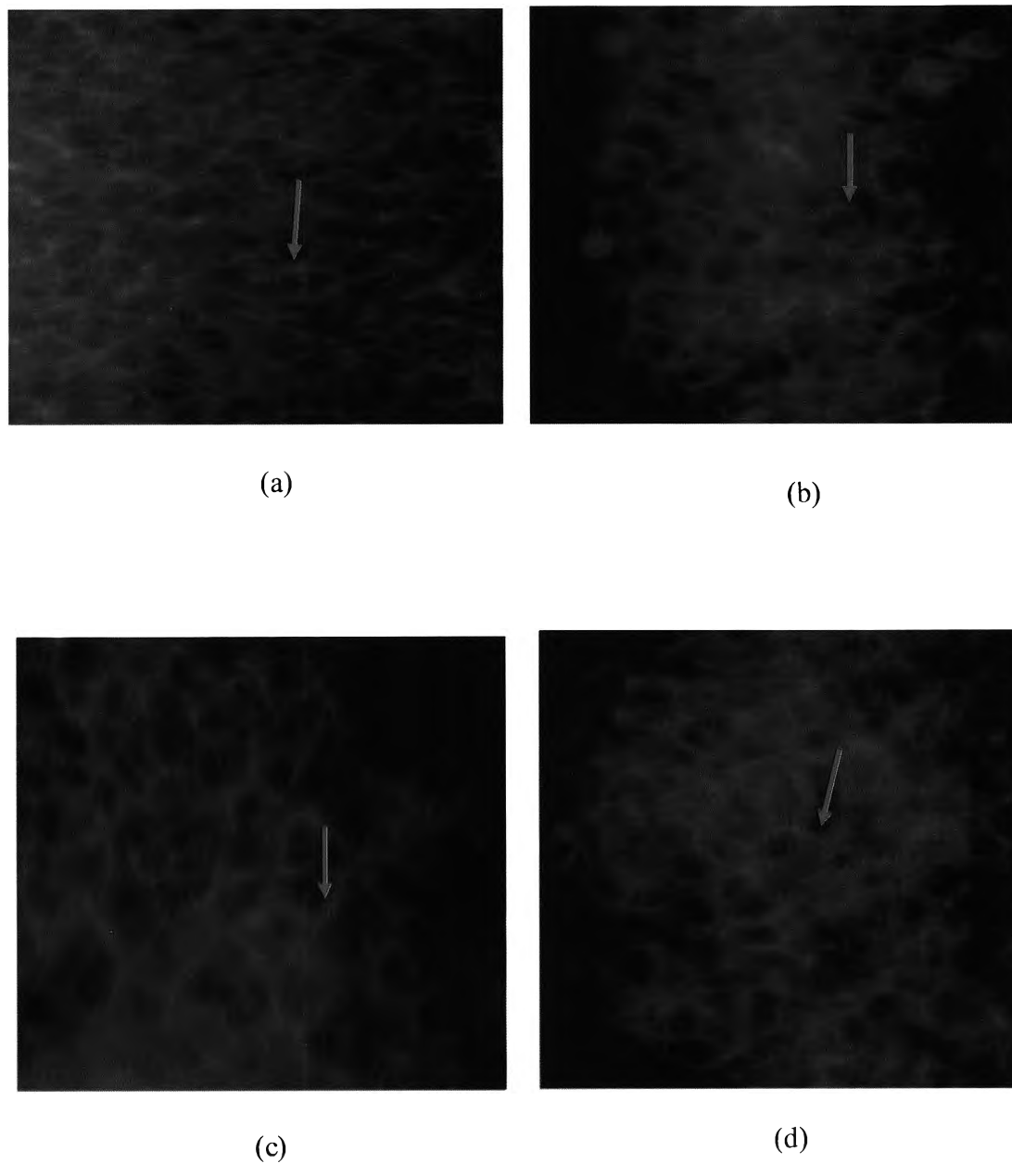
6.2 Permeation study

Human nasal epithelial cells (passage 8-10) in culture grew to a confluent monolayer within three to six days after seeding at a density of 4 x 10^5 cells/cm^2.

The development of tight junction completed within 2-3 days was confirmed by positive fluorescein isothiocyanate (FITC)-phalloidin staining. Figure 26 (a-d) illustrtate that all types of pectin could not open the tight junction which agreed with the previous research about low methoxylated pectin for nose to brain delivery (Charlton et al.,2007).

The transepithelial electrical resistance (TEER) of the confluent monolayers (Figure 38 a) reached 108.67 ± 3.93 Ω·cm^2 (n=6), 110.71 ± 7.25 Ω·cm^2 (n=7), 108.14 ± 9.62 Ω·cm^2 (n=7), and for PP pectin, HM pectin and LM pectin respectively (Figure 32). After the transport experiments, the TEER value was found to be only slightly lower, namely 104.33 ± 4.04 Ω·cm^2 (n=6), 103.67 ± 4.73 Ω·cm^2 (n=7) and 100.40 ± 5.90 Ω·cm^2 (n=7) for PP pectin, HM pectin and LM pectin respectively. TEER value of before and after experiment for 48 hours were not significantly different ($p<0.05$: ANOVA) thus these results were consistent with Figure 38. The TEER of nasal epithelial carcinoma cell cultured on porous membranes were in the same range of previous report. (Boucher et al.,1987).

Although the tight junction did not open, the permeation of BSA from all obtained spray dried microparticle through nasal cell monolayer was detected. These results may be due to the other transport mechanisms of protein. It has been reported that the potential transport mechanisms most often cited for protein and peptide in respiratory epithelia involve endocytosis and transcytosis processes (Rodman et al.,1990; Johnson et al.,1993). The microparticles at BSA:pectin ratio of 1:3 was used in the permeation experiment due to the highest protein content. The permeation of BSA through nasal cell monolayer from the microparticles as shown in Figure 37 ranking PP pectin > HM pectin > LM pectin as



(a) (b)

(c) (d)

Figure 36 Actin staining of the nasal cell monolayers after permeation experiments with microparticle formulation (400x) (a) blank monolayer (b) cell monolayers after experiment with formulation of LM pectin (c) cell monolayers after experiment with formulation of HM pectin (d) cell monolayers after experiment with formulation of Pectic polysaccharide

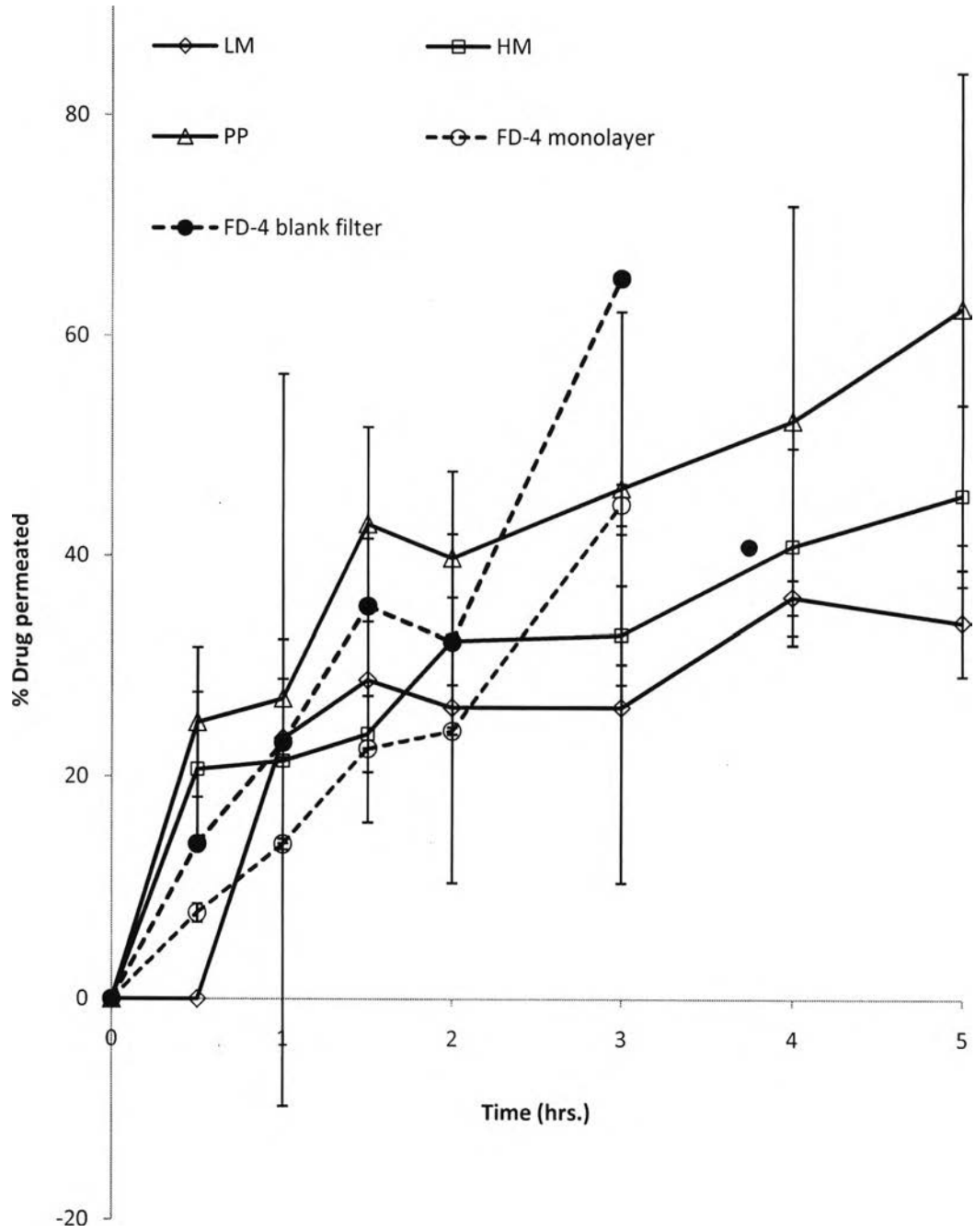


Figure 37 The permeation profiles of pectin microspheres at BSA:pectin ratio of 1:3 and FD-4 through nasal cell monolayer (--o--) and FD-4 through blank filter (-----)

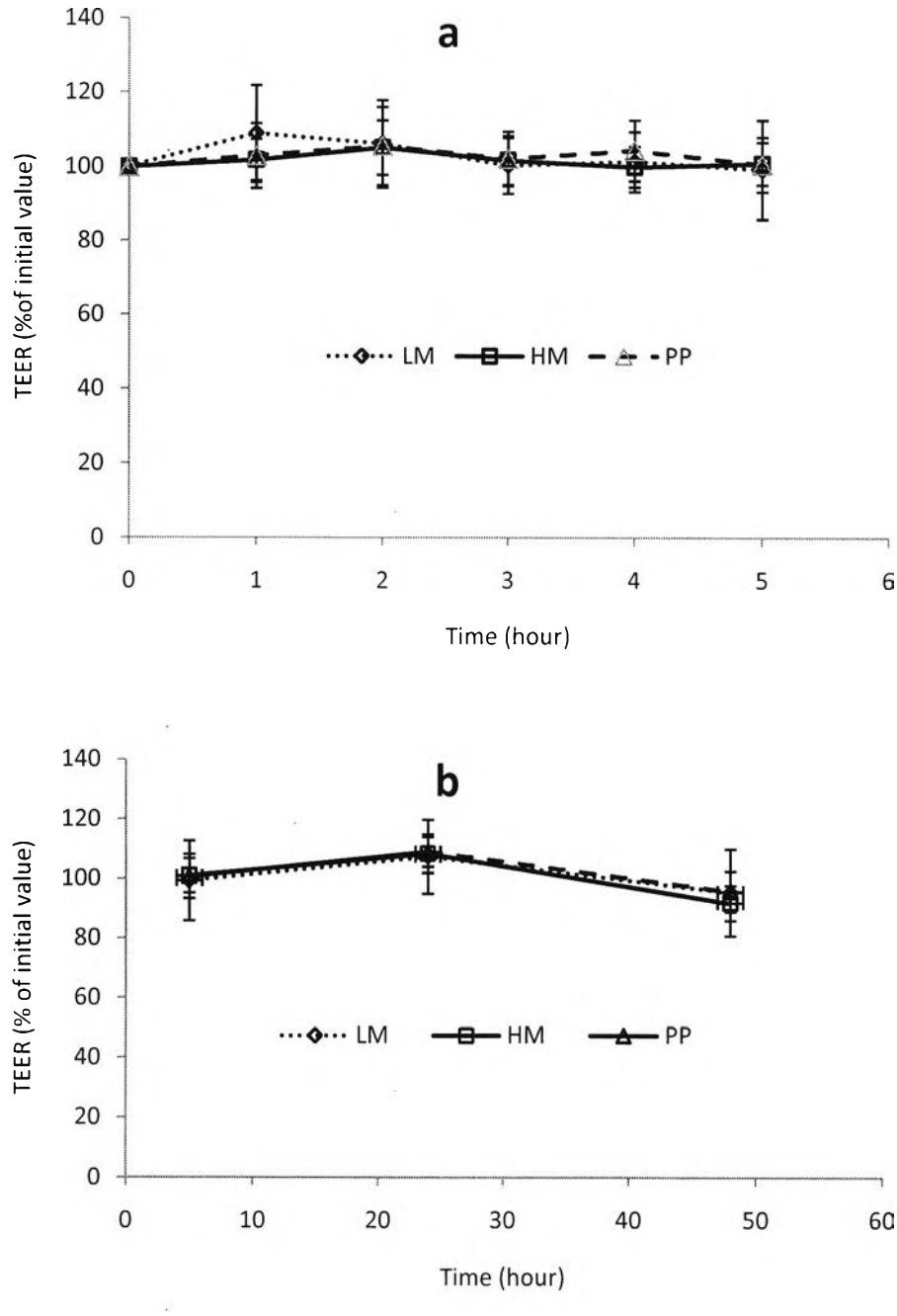


Figure 38 Effect of microspheres on the TEER of nasal cell monolayers. A: TEER of nasal cell monolayers during permeation experiment B: TEER from after permeation experiment

6.3 Cell viability

The results from trypan blue dye exclusion and haemocytometer showed the cell viability after exposure to BSA loaded pectin microparticles at all types of pectin (Table 7) for 5 hours of permeation experiment. There was no significant difference of cell viability from all types of pectin ($p>0.05$: ANOVA). These results correlated with the results of cytotoxicity study.

Table 7 Cell viability after permeation experiment of all types of pectin (n=4)

Types of pectin	PP	HM	LM
Cell viability (%)	79.32 ± 3.85	82.13 ± 5.21	81.87 ± 7.20